Causal Neural Mechanisms for Decision Making: Putting Rules into Context

Thesis submitted by:

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General Abstract

Episodic memory, an important cognitive process in humans, relies on both contextual and temporal information to recall sequences of events accurately. While nonhuman animals are capable of understanding rules based on context and have shown capability in learning temporal sequences, the ability to flexibly shift between these types of information has yet to be demonstrated. This thesis aimed to explore the cognitive mechanisms behind adapting memory sequences to changing contexts in three species. Following work in Lister hooded rats, the study was extended to New world (Common marmosets) and Old world monkeys (Rhesus macaques) to assess each species' proficiency in learning context-guided sequences. In addition, a non-invasive technique called transcranial ultrasound stimulation (TUS) was employed with the macaques to investigate potential underlying neurobiological pathways. The task design, consistent across all three species, involved learning visual object sequences (e.g., A-B or C-D) where the correct choice was contingent on one of two background contexts (e.g., blue or yellow). Results showed that rats and marmosets were proficient in learning sequences that remained constrained to a singular context but faced challenges with sequences involving a mid-trial contextual shift. Conversely, the macaques quickly mastered both fixed and context-switching sequences, facilitating further investigation into potential neurobiological mechanisms using TUS. Prior research suggests a possible role for the prefrontal-hippocampal circuitry in context-dependent learning, prompting us to apply targeted modulation to the hippocampus and prefrontal cortex separately. Notably, TUS of the anterior hippocampus enhanced performance during the initial stages of learning. In contrast, TUS of the prefrontal cortex enhanced performance in the later phases, particularly in trials that required a context shift mid-trial. Overall, this research highlights the evolutionary foundation of flexible learning and offers insights into neural modulation in primate cognition, particularly in how the brain adapts dynamically to contextual shifts when guiding memory sequences.

Dedications

To Grandma Wales, I dedicate this thesis to you.
You would have laughed at the size of it, and cried at the weight of it,
but you would have been so proud to show everyone
that walked through the door.

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Slater, B., Petkov, C.I. & Easton, A. (2025) 'Temporal context-guided memory sequencing capabilities in rodents', *Scientific Reports*, 15, 18753.

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Chapter 1: General Introduction

1.1. Overview

This thesis focuses on how animals utilise memories of prior experiences to determine the correct action to execute when exposed to a specific context. In particular, it examines the roles of the prefrontal cortex and hippocampus, both as independent and interdependent components, in this process. The research includes studies conducted in rodents and primates, encompassing both New World and Old World monkeys, utilising a context paradigm tailored to the unique characteristics of each species. Given that rodent and human studies currently dominate the field, nonhuman primates (NHPs) offer a crucial translational bridge by sharing key anatomical and functional features with the human brain while still being practical to study experimentally. By including both rodent and primate models, this thesis seeks to map the evolutionary continuity and divergence of memory-related processes across species. Additionally, this thesis explores the potential mechanisms underpinning these processes and evaluates the validity and translatability of using nonhuman species to understand the functional interplay between the hippocampus and prefrontal cortex in humans.

1.2. Episodic-like memory

Many animals can react to their environment; however, making a decision requires more than just reaction – it involves comprehension of the current environment, planning an action, and executing one or more steps to achieve a desired outcome. Decision making can be defined as the result of integrating multiple inputs to decide on an appropriate action (Gold & Shadlen, 2007).

In the field of neuroscience, decision making is explored through various perspectives across a variety of species; however, the tasks employed, as well as the specific aspects of decision making being studied, often vary considerably, complicating direct comparisons. For example, when comparing rodents with nonhuman primates, differences in cognitive complexity are a key factor influencing study design. Nonhuman primates are typically tested in more complex scenarios involving social interactions or involving more complicated, lengthy tasks that require abstract reasoning (Rilling & Sanfey, 2011; Mansouri et al., 2020), whereas rodent studies tend to focus on simpler designs centred around associative learning and implicit fear

mechanisms. These rodent models are highly tractable and allow for invasive manipulation which offer invaluable tools for investigating the neural mechanisms underlying decision making (Hanks & Summerfield, 2017). Such findings can then inform studies in higher-level species, which explains why these rodent tasks are deliberately kept ‘simpler’.

A prominent area of study in decision making is memory-based decision making, which relies on retrieving information from memory to guide choices (Weilbächer & Gluth, 2016). In humans, this is closely associated with episodic memory, which has also been investigated across various species through use of tasks such as the ‘What-Where-When’ paradigm (Crystal, 2018). Episodic memory was introduced by Tulving (1983) and defined as a long-term repository for event-specific memories that integrate information about “what” occurred, “where” it happened, and “when” it took place within a distinct spatial and temporal framework.

While both episodic and declarative memory are part of the explicit memory system, they differ in scope and function. Declarative memory encompasses the conscious recall of facts and events and is divided into two main components: semantic memory and episodic memory. Semantic memory involves general knowledge and facts about the world, such as knowing that Paris is the capital of France. In contrast, episodic memory is concerned with personal experiences and events tied to specific times and places, allowing individuals to mentally “travel” back in time to relive these experiences. Episodic memory integrates details about "what" occurred, "where" it happened, and "when" it took place, giving it a more personal and contextualised character (Tulving & Markowitsch, 1998).

Episodic memory is a subset of declarative memory, providing a more specific and personalised recollection of past events. The distinction between these memory systems is not just theoretical but also neurobiologically significant. While declarative memory encompasses both semantic and episodic components, episodic memory is particularly associated with structures like the hippocampus, which is crucial for encoding and retrieving event-specific memories.

The capacity for episodic memory, however, remains a subject of considerable debate when studying nonhuman animals. Episodic memory is often linked to higher-order cognitive abilities such as self-awareness or mental time travel – the ability to mentally

project oneself into the past or future (Clayton et al., 2003; Suddendorf & Busby, 2005; Suddendorf & Corballis, 2007). Consequently, researchers generally refer to 'episodic-like memory' in nonhuman animals, acknowledging that while animals may not possess the same subjective experience of episodic memory as humans, they can still demonstrate the ability to integrate "what", "where," and "when" information to guide their decisions . As such, the fundamental process of using memories to inform decisions is shared between episodic-like memory in animals and episodic memory in humans (Easton et al., 2012).

Humans frequently rely on episodic memory to inform future actions (Shadlen & Shohamy, 2016; Duncan & Shohamy, 2016). For example, deciding which route to take while driving in a familiar city involves recalling specific details, such as traffic congestion, rush hour patterns, or construction works. Such decisions depend on the ability to use context, both spatial and temporal, to retrieve and apply past experiences effectively. Understanding how episodic memory and contextual factors contribute to decision making in humans and other species provides a valuable framework for identifying the shared and unique mechanisms involved.

1.3. Context

In its fundamental form, the term context refers to a collection of factors that define an event, more specifically, any set of cues which situate a person or animal in place and time (Nadel, 2008). Taken from studies conducted in humans, the list of factors deemed to be important is exhaustive, including physical items, surrounding environments, physiological and emotional processes as well as spatial and temporal components (Robertson et al., 2015). Which factors are crucial in the contextualisation of a memory episode, is unclear, and perhaps irrelevant. This is because the context of an episode may include any or all of the factors mentioned above, each of which can be encoded during memory formation, though only one may be necessary for later recollection of the event (Easton et al., 2024). The use of context appears to be an important mechanism in episodic memory, serving to reduce interference between similar memories and enabling humans to recall the richly interconnected tapestry of their lives.

Rudy and O'Reilly (1999) outlined in their review that contexts are both dynamic and independent of the observer. Using the example of a spatial location – a common test

of contextual understanding in animal studies – they describe how ‘an office’ can serve as a context. Regardless of whether the observer is present in the office or whether items within it are rearranged, the space remains an office. To illustrate the dynamic nature of context, they note that if the office furniture (e.g., desk, computer, chair) is replaced with a sofa, television and coffee table, the context shifts to resemble that of a living room.

There is, unfortunately a complication in this example, which Rudy and O’Reilly also acknowledge. Humans have a strong affinity for semantic labels, enabling them to recognise the room as still being an office, albeit with different, slightly unusual furniture. Hyman et al. (2012) argue that contexts can be abstract, and in such cases, may rely on human language to be discernible. They further emphasise the importance of spatial and sensory stimuli surrounding an individual, and in nonhuman animal studies, researchers often manipulate the spatial and temporal components to investigate context-dependent mechanisms within the brain, particularly in rodents (Rudy, 2009).

For humans, contexts are easily and readily understood, as we are explicitly taught the importance of behaving appropriately depending on the social context we are in. From an early age, individuals learn to adjust their actions according to who is present, what time it is, or what the setting demands. These contexts can include physical surroundings, social conventions, or abstract norms, and often interact in complex ways to guide decision-making.

To illustrate this, consider the scenario depicted in Figure 1.1. In Figure 1.1A, an individual arrives at work and simply determines whether anyone is present before deciding to greet colleagues or proceed directly to work. This represents a relatively straightforward context-based decision driven by a single cue: social presence. In Figure 1.1B, a second factor is introduced – whether the individual is late – adding an additional layer of contextual complexity. The decision to greet others now depends not only on who is present but also on temporal information, demonstrating how multiple, interacting cues can dynamically shape behaviour. This example mirrors how humans seamlessly integrate various contextual elements in daily life to guide appropriate responses.

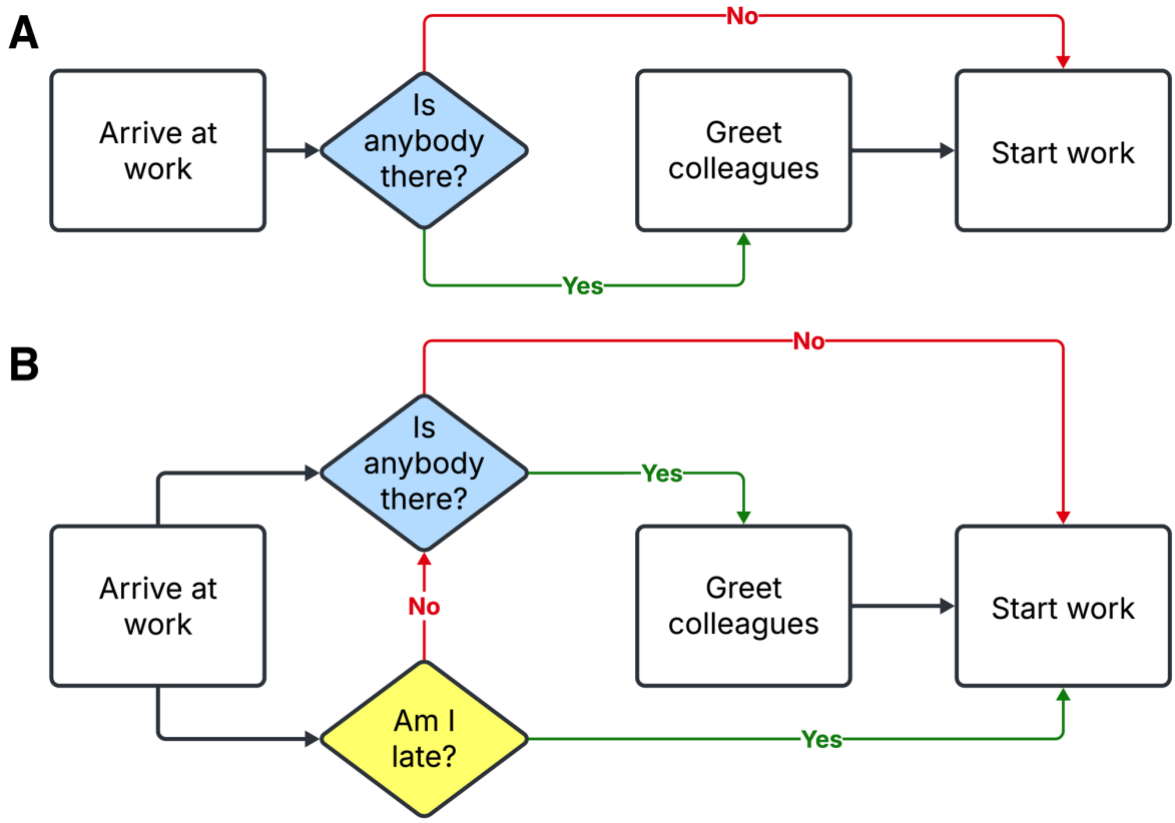


Figure 1.1. Illustration of contextual complexity in everyday decision-making.

A) *A simple decision-making flowchart involving a single contextual factor – social presence. Upon arriving at work, the individual checks if anyone is present; if so, they greet colleagues before starting work, otherwise they proceed directly to work.* ***B)*** *An expanded version incorporating a second contextual factor – time of arrival. If nobody is present, the individual then evaluates whether they are late. If they are late, they skip greetings and start work; if not, they loop back to check again for others' presence, introducing a context-dependent feedback loop. This illustrates how additional contextual information can modify behavioural sequences and increase decision complexity.*

While the human ability to interpret and navigate such complex contexts is often explicit and scaffolded by language and social learning, nonhuman animals also demonstrate context sensitivity, albeit typically in more implicit or ecologically relevant settings. For example, in a study with rats, the rewarded object in a pair of familiar objects differed based on the context encountered (Figure 1.2A). In context 1, the reward was located under object A, whereas in context 2, object A did not yield a reward (Navawongse & Eichenbaum, 2013; Place et al., 2016). The rats quickly

learned to use the context as a cue to determine where they should search to collect the reward (Figure 1.2B).

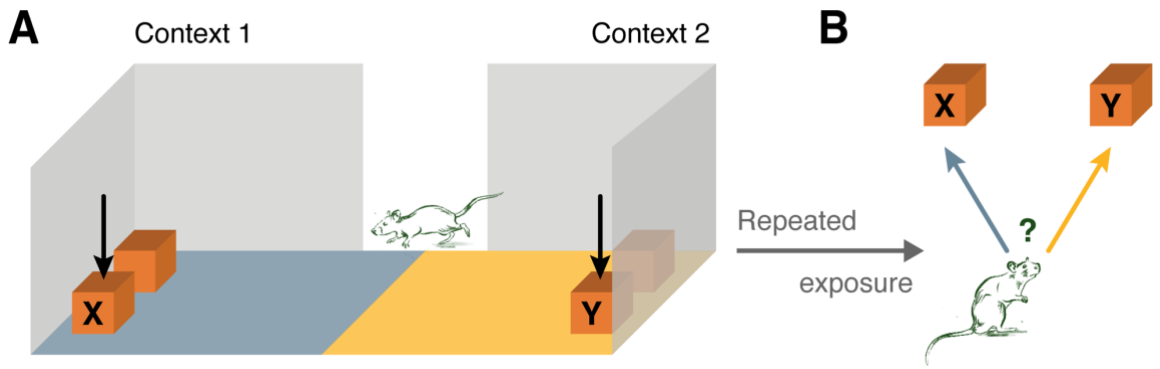


Figure 1.2 Context-dependent object discrimination in rats, adapted from Navawongse and Eichenbaum (2013).

A) *Schematic of the experiment. Rats were exposed to two distinct spatial contexts (Context 1 and Context 2), each containing a pair of objects. In Context 1, object X was rewarded, while in Context 2, object Y was rewarded. Contextual cues (e.g., floor colour and spatial geometry) differentiated the environments. B) In the testing phase, both objects (X and Y) were presented in the same location in each context. The rat's ability to select the previously rewarded object was dependent on which context had been experienced prior to the test. With repeated exposure, rats learned to use the contextual information to guide object choice, demonstrating flexible, context-sensitive memory.*

In contrast, the study of context-dependent memory in nonhuman primates has been relatively sparse, and when context is examined, it is often less tangible than in rodent studies. Gaffan (1994) made a significant contribution by using an automated system with visual displays on flat screens, presenting static scenes where monkeys interacted with objects embedded within differing backgrounds. This setup allowed for precise control over the environment, minimising experimenter influence and enhancing reproducibility. The task required monkeys to use contextual information from these scenes to guide object choices. Although the task focused on object and scene memory, it demanded the integration of both object identity and environmental context, which resembles context-dependent memory.

The framework developed by Gaffan (1994) included several variants, such as place discrimination tasks where monkeys distinguished objects based on spatial location

within a scene, and object-in-place discrimination, where monkeys had to remember the specific arrangement of objects within a scene. This task approximated episodic-like memory by requiring the animals to integrate ‘what,’ ‘where,’ and ‘context’ within a single memory.

Despite these innovations, the study of context in nonhuman primates has remained limited, especially when compared to the rich, tangible manipulations common in rodent studies.

In nonhuman primates, the use of context is generally less explicit, with studies often favouring abstract rule-learning or episodic memory tasks over strictly context-dependent rules. This preference may stem from practical challenges, as the larger size of primates makes it difficult to manipulate their physical environment in a way that effectively assesses contextual shifts. And yet, as demonstrated by Gaffan (1994), context – or a form of context – can be successfully represented using a screen.

The limited emphasis on context-dependent tasks in primates may change with the growing accessibility and adaptability of virtual reality (VR) technologies. While VR has already been employed in studies involving rodents (Thurley & Ayaz, 2017) and primates (Dolins et al., 2017), its ability to fully replicate and serve as a true context remains an open question (Wälti et al., 2019).

1.4. Prefrontal Cortex

The prefrontal cortex is a brain region critical for a wide array of cognitive functions, including decision making, episodic memory and sequential behaviour. The term ‘prefrontal’ itself, however, is somewhat misleading, as anatomically, there are no brain structures that precede the frontal cortex (Fuster, 2015). Instead, the prefrontal cortex refers to the most anterior sector of the frontal cortex in mammalian animals. Additionally, there is no consistent definition of the prefrontal cortex, with different nomenclatures being used to delineate this region. This inconsistency is particularly relevant when comparing studies across species as the prefrontal cortex in rodent and primates is not directly analogous, as will be discussed in further detail later (Figure 1.3 A-C).

Publications on the prefrontal cortex have been increasing since 1990 (Laubach et al., 2018), with researchers using various definitions of the term ‘prefrontal cortex’ to

describe frontal brain areas in both rodents (mice and rats) and primates (monkeys and humans). In primates, the prefrontal cortex is often described using both the Brodmann areas (BA) and topographical regions it encompasses (Carlén, 2017). In humans, the prefrontal cortex is thought to span from BA8 to 14 and BA44 to 47 (Öngür et al., 2003). It is commonly referred to as the 'medial' or 'lateral' prefrontal cortex, but it can be further divided into dorsal and ventral portions too. In rodents, however, the distinction is less precisely defined and is typically referred to as just the 'prefrontal cortex' or 'medial prefrontal cortex', with no further subdivisions (Figure 1.3A). As such, miscommunication between inter-disciplinary studies is a major risk.

This ambiguity partly stems from confusion about which brain areas anatomically define the rodent prefrontal cortex. In a comprehensive review, Laubach et al. (2018) propose that this controversy arises from inconsistent use of atlas references and the introduction of novel naming conventions by different laboratories, each offering its own definition for the prefrontal areas under investigation.

Cortical regions are typically classified based on the structure and prominence of layer 4. Homotypical cortex exhibits a proportionate and well-defined laminar organisation; agranular cortex lacks a discernible layer 4; granular cortex, typical of primary sensory areas, features a markedly enlarged layer 4; and dysgranular cortex presents with a visible but underdeveloped layer 4. Within this framework, the rodent prefrontal cortex is generally considered anatomically homologous to the medial prefrontal cortex in primates. It encompasses the anterior cingulate cortex (BA24), the infralimbic area (BA25), the prelimbic area (BA32), and parts of the orbitofrontal cortex (BA10) (Heukelum et al., 2020; Bizon et al., 2012). This proposed homology is supported by cytoarchitectonic evidence indicating that the rodent prefrontal cortex is 'agranular' and lacks a distinct layer 4 (Vogt et al., 2005; Wise, 2008). In contrast, in primates, this absence is largely confined to medial prefrontal regions, whereas lateral areas retain a well-developed layer 4, and as such are classified as 'granular' (Werd et al., 2010).

Although this anatomical equivalence supports a medial prefrontal homology, it has been challenged by behavioural and functional evidence suggesting that the rodent prefrontal cortex may also parallel aspects of both the ventromedial or dorsolateral prefrontal cortices in primates. Indeed, rodent prefrontal activity exhibits functional

characteristics attributed to both medial and lateral regions of the primate prefrontal cortex (Uylings et al., 2003; Brown & Bowman, 2002; Chudasama, 2011).

Nonetheless, the extent to which these parallels hold remains debated and has yet to be fully accepted by the community.

In terms of functionality, the medial prefrontal cortex is similar across rodents, nonhuman primates and humans (Schaeffer et al., 2020). Among nonhuman primates, the common marmoset (*Callithrix jacchus*), a New World monkey species, is increasingly being adopted as an alternative to the more commonly studied rhesus macaque (*Macaca mulatta*). This shift is driven by the marmoset's smaller size, which makes them easier to house and more cost-effective than their Old World counterparts. Notably, marmosets possess a granular lateral prefrontal cortex, a feature shared with macaques but believed to be absent in rodents (Burman et al., 2006).

In a study comparing intrinsic functional clustering across rats, marmosets, and humans, Shaeffer et al. (2020) found that while the medial prefrontal cortex is conserved across all three species, its organisation differs considerably. Specifically, rat medial prefrontal connectivity shows the greatest similarity with premotor regions, unlike primates (both marmosets and humans), where the medial prefrontal connectivity is more interconnected with dorsolateral prefrontal regions (Figure 1.3B-C).

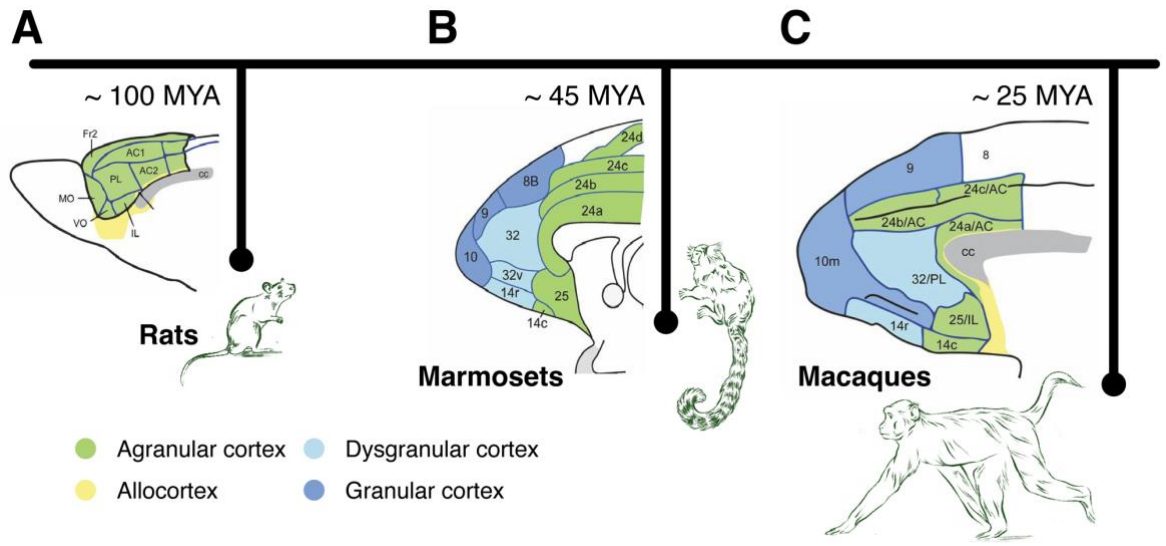


Figure 1.3 Evolutionary expansion and differentiation of the prefrontal cortex across rats, marmosets, and macaques.

A) *Schematic representation of the rat prefrontal cortex, illustrating the arrangement of agranular, dysgranular, and allocortical regions.* ***B)*** *Marmoset prefrontal cortex, showing the emergence of more defined dysgranular and early granular regions.* ***C)*** *Macaque prefrontal cortex, depicting a further elaboration of granular prefrontal fields, including areas 9 and 8, associated with higher-order cognitive processes. Coloured regions indicate distinct cortical types: agranular cortex (green), allocortex (yellow), dysgranular cortex (light blue), and granular cortex (blue). The approximate divergence times shown above each schematic (~100 million years ago (MYA) for rats, ~45 MYA for marmosets, and ~25 MYA for macaques) represent the estimated points at which humans last shared a common ancestor with each species. Abbreviations correspond to key cortical subdivisions commonly referenced in comparative neuroanatomy: AC = anterior cingulate; cc = corpus callosum; Fr2 = frontal area; IL = infralimbic; MO = medial orbital; PL = prelimbic; VO = ventral orbital. Adapted from Roberts & Clarke, 2019.*

As such, the remaining discussion will focus specifically on the medial prefrontal cortex across species, as this region is consistently implicated in context memory, episodic-like memory, and decision making in rodents, nonhuman primates and humans (Euston et al., 2012; Rushworth et al., 2011). The medial prefrontal cortex is thought to support memory and decision making through ‘top-down’ control of memory processing (Dobbins et al., 2002; Navawongse & Eichenbaum, 2013; Farovik et al., 2008; Eichenbaum, 2017). For example, patients with prefrontal damage show significant impairments when required to ignore interference from competing

memories, such as relearning new associations for familiar items, i.e., verbal word pairs (Shimamura et al., 1995).

Furthermore, studies across species suggest that the medial prefrontal cortex plays a role in both remote and recent memory recall, with an emphasis on remote memory recall requiring greater cognitive control. This transition is thought to involve the medial prefrontal cortex taking control over the hippocampus during memory retrieval (Frankland & Bontempi, 2005; Takashima et al., 2006; Nieuwenhuis & Takashima, 2011). The prefrontal cortex is thought to exert this control through projections to inhibitory neurons in various cortical regions, including the medial temporal lobe (Anderson et al., 2016). Indeed, the hippocampal formation primarily innervates the medial prefrontal cortices (specifically, areas 32 and 25) , with lesser projections to orbital, and even fewer to lateral prefrontal cortices (Barbas & Blatt, 1995). This connectivity highlights the critical role of the medial prefrontal cortex in memory processing, particular in resolving interference or overlapping memories during decision making.

1.5. Hippocampus

Ever since the removal of the medial temporal lobe in patient H.M. caused the individual to suffer from anterograde amnesia (the inability to create new memories), the hippocampus has been considered to be an integral component in the brain's mechanism of forming and storing recent memories (Scoville & Milner, 1957). Many animal studies have since displayed the time-dependent involvement of the hippocampus in memory consolidation with hippocampal damaged humans (Spiers et al., 2001), nonhuman primates (Forcelli et al., 2014), and rodents (Winocur et al., 2013), failing to form and recall newly acquired memories.

Anatomically, the hippocampus is comprised of several regions, each with unique functions and connectivity patterns. The main regions include the dentate gyrus, the cornu ammonis and the subiculum. These are cytoarchitectonically defined and generally conceived as being arranged in a canonical circuit, also known as the 'trisynaptic circuit'. The trisynaptic circuit is characterised, as the name suggests, by three sequential synaptic connections that propagate the flow of information from neurons in the entorhinal cortex, a region in the neighbouring parahippocampal gyrus, to the dentate gyrus and onward to several subdivisions of the cornu ammonis.

Following the circuit from its origin (Zeidman & Maguire, 2016), neurons in the entorhinal cortex, project to granule cells within the dentate gyrus located in the central portion of the hippocampus. These granule cells project to the third subdivision of the cornu ammonis (CA3) via mossy fibre axons, synapsing onto pyramidal neurons within CA3. The final stage of the circuit involves these CA3 pyramidal neurons projecting their axons, known as Schaeffer collaterals, to other pyramidal neurons located in the first subdivision of the cornu ammonis (CA1). This connection is essential for the integration of information processed in CA3 and its transmission to CA1, which serves as the primary output of the hippocampus (Lee et al., 2020).

These trisynaptic loops were originally perceived as being present throughout the entire hippocampus and were thought to be confined at the cross-section or 'lamella' as independent modules (Andersen et al., 1971). This 'lamellar hypothesis' has since been updated with anatomical studies, demonstrating these modules as not functionally independent, instead revealing extensive longitudinal and transverse connectivity along and across the entire hippocampus (Knierim, 2015; Sloviter & Lømo, 2012). Additionally, the second subdivision of the cornu ammonis (CA2), once considered a transitional zone, is now recognised as having its own unique functions, comparable in importance to CA1 and CA3 (Pang et al., 2019).

The intrinsic organisation of the hippocampus is widely recognised as being conserved across species, including rodent, nonhuman primates, and humans (Burwell, 2000; Strange et al., 2014). This conservation underpins the consistent nomenclature of intrahippocampal regions across species. Along its longitudinal axis, the hippocampus can be divided into anterior and posterior portions in primates (including humans, ***Figure*** 1.4), corresponding to the ventral and dorsal portions in rodents respectively (Moser & Moser, 1998). Notably, the orientation and structure of this axis differ between rodents and primates (***Figure*** 1.4). In primates, the anterior hippocampus is significantly larger and flatter, occupying more of the frontal space within the anterior medial temporal lobe compared to rodents (Strange et al., 2014). This disproportionate orientation of the hippocampus between species is thought to influence the hippocampus' connectivity with the surrounding neocortex, particularly as non-sensory inputs to the hippocampus have progressively increased in parallel with the enlargement of the neocortex during mammalian evolution (Buzsáki et al., 2013).

Structural variation and distinct connectivity patterns along the hippocampal longitudinal axis have led to ongoing debate about the specific functional specialisations of its anterior and posterior regions (Poppenk et al., 2013). While there is general consensus across species that the posterior hippocampus is more engaged in visuospatial processing and the anterior hippocampus in olfactory, visceral and gustatory functions (Fanselow & Dong, 2010), the degree to which these specialisations hold – particularly in less extensively studied species such as nonhuman primates – remains to be fully justified.

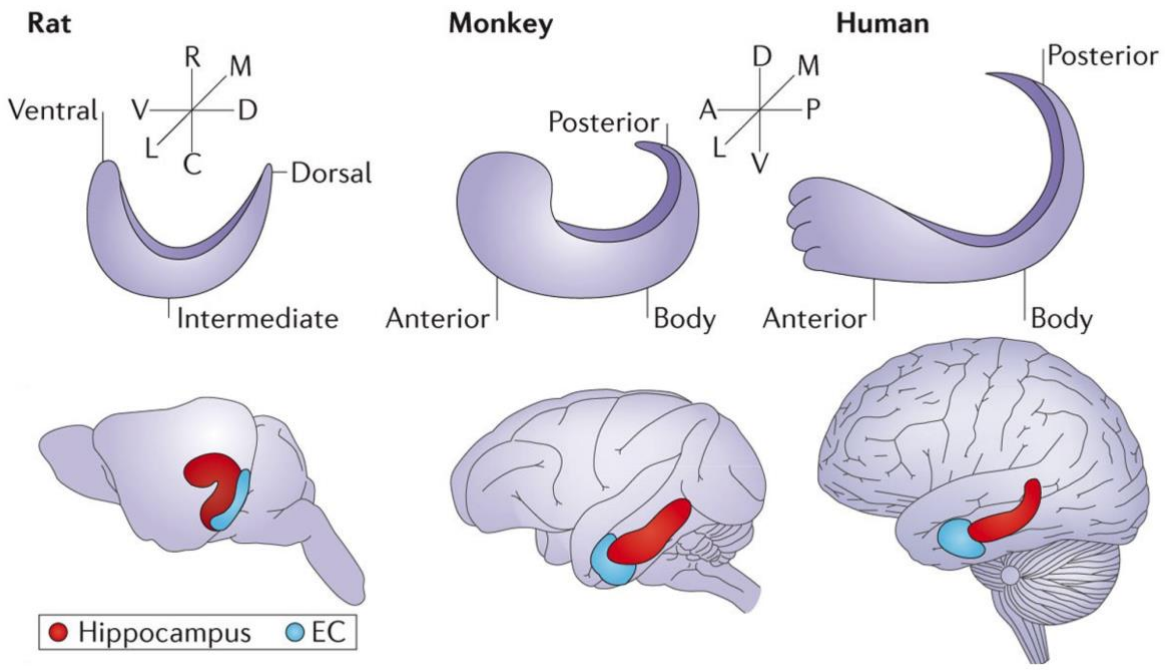


Figure 1.4. Comparison of the longitudinal axis of the hippocampus between primates and rodents.

The hippocampal orientation as seen in rats, macaque monkeys and humans, with the position within the brain (red) alongside the entorhinal cortex (EC – blue). Abbreviations: A = anterior; C = caudal; D = dorsal; L = lateral; M = medial; P = posterior; R = rostral; V = ventral. Taken from Strange et al. (2014).

These differences in hippocampal orientation and function are mirrored in the organisation of the neocortex. In rodents, the majority of neocortex is dedicated to processing sensory inputs and generating motor outputs. In contrast, in primates, a distinctly larger proportion is responsible for higher-order processes, such as decision-making and abstract reasoning. This functional distinction is reflected in hippocampal

connectivity: in rodents, the dorsal hippocampus is proportionally larger than the ventral portion and receives predominantly sensorimotor inputs. Conversely, in primates, the anterior hippocampus is more extensive and frontally positioned, receiving greater input from higher-order regions, such as the prefrontal cortex (Buzsáki & Tingley, 2018).

Hippocampal connectivity to the entorhinal cortex also reflects distinct differences across species. For example, due to rodents' strong reliance on smell and emphasis on olfactory processing, evidence shows that olfactory inputs are distributed across the entire entorhinal cortex in rats. In monkeys, however, this proportion drops to just 15 % and becomes even sparser in humans (Insausti et al., 2002; Basile et al., 2020). These variations emphasise how sensory priorities shape the architecture of the hippocampus and its connections, reflecting the diversity in how different species process their environment.

Another important difference along the longitudinal axis, is the distribution and size of place cells. Place cells are specialised neurons located in the hippocampus that become active when an animal is in a specific location within its environment (O'Keefe, 1976; Nadel, 2008). The dorsal hippocampus (posterior in primates) is characterised by a high density of place cells with smaller place fields approximately one metre in size (Kjelstrup et al., 2008; Fanselow & Dong, 2010). Research suggests that place cells located in the dorsal hippocampus exhibit a high degree of spatial specificity and fine-grained resolution, enabling the detailed encoding of spatial information and objects within an environment. In contrast, the ventral hippocampus (anterior in primates) contains place cells that have much larger place fields (up to 10 metres in size) and exhibit less spatial specificity as a result (Kjelstrup et al., 2008; Jung et al., 1994). This had led to the idea that the ventral hippocampus is more involved in processing contextual and emotional information than precise spatial mapping.

The differences in place cell properties along the longitudinal axis of the hippocampus reflect a functional gradient, with the dorsal (posterior) hippocampus encoding detailed spatial information, and the ventral (anterior) hippocampus integrating this information with emotional and contextual cues, highlighting their combined importance in episodic memory. In support of this, Keinath and colleagues (2014) argue that despite differences in spatial scaling, ventral populations maintain spatial resolution

comparable to dorsal populations, by encoding a distributed representation of space. They suggest this shift in representational coding may help balance the competing demands of memory interference and generalisation, supporting adaptive memory functions. Furthermore, Maurer and Nadel (2021) propose that the hippocampus achieves this by employing a peristaltic-like mechanism that continuously moves contextual information from the dorsal (posterior) to the ventral (anterior) hippocampus via recurrent networks. This mechanism plausibly explains the hippocampus' ability to facilitate smooth transitions between contexts. Supporting evidence from rodent studies shows that the hippocampal network dynamically shifts between contextual representations during exposure to multiple environments. For example, Jezek et al. (2011) demonstrated that when rats were exposed continuously to two distinct contexts, hippocampal activity alternated between representing the two contexts before eventually stabilising on the context in which the rats remained.

Tracer studies in animal models have revealed that there is little to no direct connectivity between the ventral (anterior) and dorsal (posterior) hippocampus. It appears, in rodents at least, that the two communicate indirectly via the entorhinal cortex through connections to the perirhinal and parahippocampal cortices (Fanselow & Dong, 2010; Poppenk et al., 2013). Indeed, in humans the strongest coupling is between the anterior (ventral) hippocampus and the perirhinal cortex whereas the posterior (dorsal) hippocampus is coupled with the parahippocampal cortex (Libby et al., 2012; Kahn et al., 2008).

1.6. Prefrontal-Hippocampal Connectivity

The prefrontal cortex and hippocampus are anatomically interconnected through direct and indirect pathways (Hoover & Vertes, 2007), and evidence suggests these pathways are conserved across rodents and primates (Eichenbaum, 2017; Rosene & Hoesen, 1977; Witter et al., 1989; Burwell et al., 1995; Lavenex et al., 2002). The primary projection, a monosynaptic connection, originates from the ventral (anterior) hippocampus and targets all medial prefrontal cortex regions, including the infralimbic, prelimbic, anterior cingulate, and orbitofrontal cortices (Jay et al., 1989; Hoover & Vertes, 2007). In contrast, the dorsal (posterior) hippocampus lacks direct projections to the medial prefrontal cortex and instead communicates indirectly via bidirectional

connections via the nucleus reuniens (Varela et al., 2014), or through the perirhinal and the lateral entorhinal cortices (Burwell & Amaral, 1998).

These projections consist primarily of excitatory glutamatergic neurons that synapse onto gamma-aminobutyric (GABA)-ergic interneurons within the medial prefrontal cortex (Jay et al., 1992). Hippocampal inputs to the medial prefrontal cortex are α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) receptor-dependent (Jay et al., 1992), while hippocampal synapses exhibit N-methyl-D-aspartate (NMDA) receptor-dependent plasticity, which is critical for learning and memory (Dudek & Bear, 1992). Furthermore, the glutamatergic projections from the nucleus reuniens (Bokor et al., 2002) suggest that it plays a modulatory role in synaptic plasticity in both the hippocampus and medial prefrontal cortex, highlighting its importance in coordinating interactions between these regions (Di Prisco & Vertes, 2006).

The nucleus reuniens is a midline thalamic nucleus with reciprocal connections to both the hippocampus and medial prefrontal cortex (Ferraris et al., 2021, Figure 1.5A), positioning it as a critical structure in a multitude of cognitive and memory processes, in particular memory consolidation (Frankland & Bontempi, 2005). Its central location justifies its role as an interface between these two brain regions, facilitating the integration of hippocampal and prefrontal connectivity. While the nucleus reuniens has been extensively studied in rodents (Figure 1.5B), it has also been identified in other mammals, including monkeys and humans (Amaral & Cowan, 1980; Hirai & Jones, 1989) . Functional data regarding the nucleus reuniens, in humans, however, remain sparse due to methodological limitations that make experimental exploration challenging.

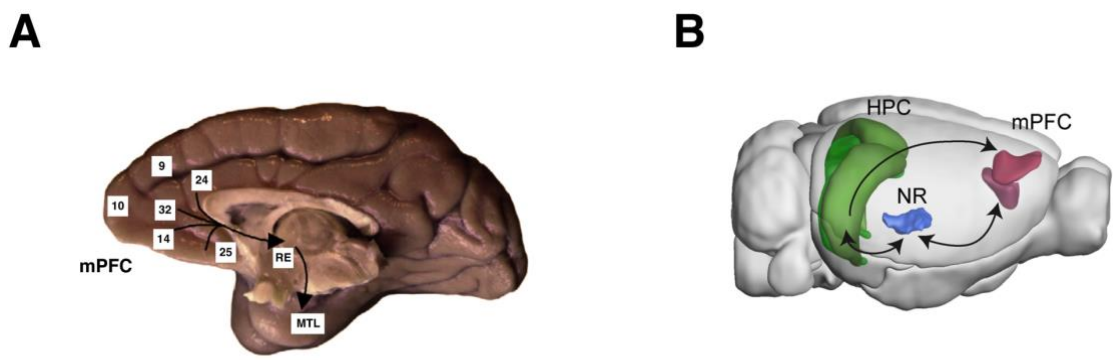


Figure 1.5. Connectivity of the nucleus reuniens in monkeys and rats.

In both species, medial prefrontal cortex (mPFC) areas that receive robust projections from the hippocampus (HPC) project to the nucleus reuniens (RE/NR), which in turn, gives rise to one of the most prominent thalamic pathways to the medial temporal lobe (MTL). ***A)*** *Schematic of connectivity in the rhesus macaque (adapted from Anderson et al. (2016)), and* ***B)*** *Schematic of connectivity in the rat (adapted from Ferraris et al. (2021)).*

In a seminal paper by Miller and Cohen (2001), it was proposed that the hippocampus and prefrontal cortex work together during episodic memory processing. The two authors described this interaction using a 'railroad' metaphor with the hippocampus laying down new tracks (encoding episodic memories) that the switchboard (the prefrontal cortex) could switch between (and select) depending on the surrounding context. Subsequent theories have all built upon this initial idea to suggest that the hippocampus is primarily responsible for organising and categorising episodic memories by context in which they were acquired and the prefrontal cortex to be responsible for selecting the relevant episodic memory upon a given contextual environment.

Preston and Eichenbaum (2013) outline a potential neuroanatomical pathway in which this mechanism might occur (Figure 1.6). They suggest that sensory information from the current contextual environment such as information about "what" is being experienced, specifically the object stimuli and familiarity of the object, is processed by the perirhinal cortex and lateral entorhinal cortex, whereas, information regarding the surrounding spatial context ("where") is processed by the parahippocampal cortex and medial entorhinal area. Both streams of information then merge before entering the medial temporal lobe, whereby they are processed by the posterior hippocampus. The hippocampal longitudinal axis then serves as a quantifier of context before information regarding a change in context is sent from the anterior hippocampus to the medial prefrontal cortex. Once the contextual information has been received, it is returned back to the hippocampus (through the indirect connections via the nucleus reuniens or entorhinal cortex) allowing for 'top down' control over memory recall and selection. This model is supported by evidence from a study carried out by Rajasethupathy et al. (2015) who were able to modulate the medial prefrontal cortex (primarily the anterior cingulate cortex) in mice using optogenetics and as a result,

successfully modulate the expression of fear-conditioned memories within the animals.

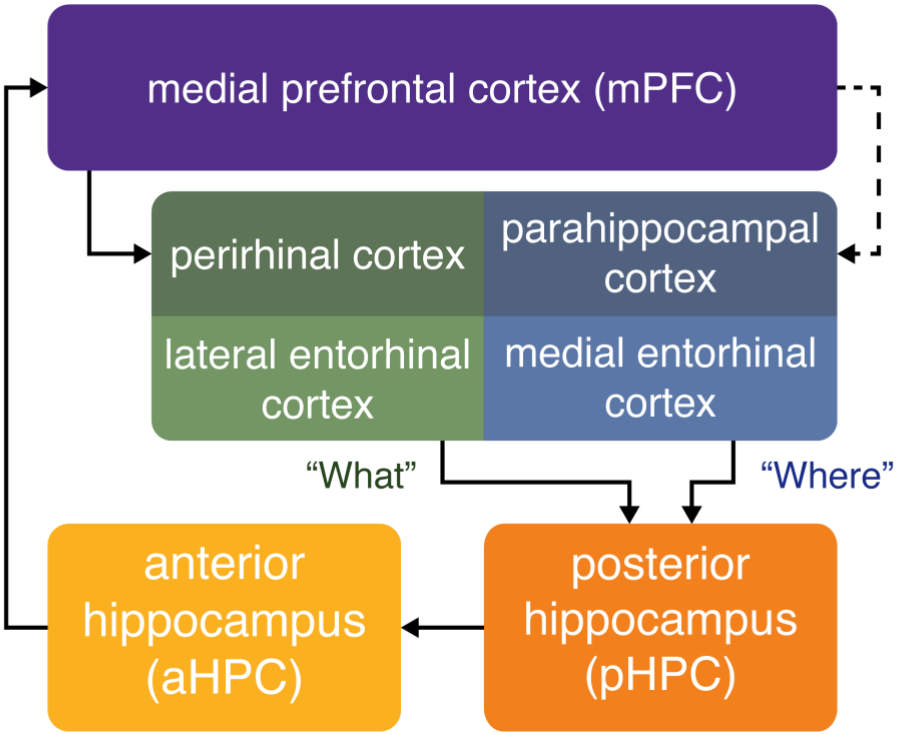


Figure 1.6. Schematic adapted from Preston and Eichenbaum (2013) illustrating proposed context-processing pathways.

Object-related ("what") information is processed via the perirhinal and lateral entorhinal cortices (green), while spatial-contextual ("where") information is processed by the parahippocampal cortex and medial entorhinal areas (blue). These streams converge in the posterior hippocampus (orange), where contextual integration occurs. The anterior hippocampus (yellow) then relays context-change signals to the medial prefrontal cortex (purple), which in turn exerts top-down influence on hippocampal memory retrieval via indirect pathways (e.g., nucleus reuniens or entorhinal cortex).

Another way in which the medial prefrontal cortex and hippocampus are thought to communicate with one another, that enables this modulatory process to occur, is through the use of theta oscillations and cross-area synchronisation. In a study by Place et al. (2016), they assessed theta oscillation synchrony between the two regions whilst rats undertook a task, where they had to remember an object-location association based on their contextual surroundings (Navawongse & Eichenbaum, 2013). It was shown that theta oscillations from the hippocampus preceded medial

prefrontal theta when animals entered the context, suggesting the flow of information was from the hippocampus to the medial prefrontal cortex (a propagation of contextual information). Conversely, when the animal approached an object and was choosing the correct item to receive a reward, the flow of information was reversed so that medial prefrontal theta now preceded hippocampal theta. This distinct switch in oscillatory behaviour was predominantly seen during correct responses highlighting the mechanism as being important in successful context-dependent memory selection (Benchenane et al., 2010). Recent work on prefrontal-hippocampal connectivity has been centred around including the nucleus reuniens (present in the thalamus) within this pathway after strong evidence suggests it is focal in propagating and synchronising theta oscillations between the two regions (Dolleman-van der Weel et al., 2019).

Schema are strongly connected networks of representations present within the neocortex (Kesteren et al., 2012), and are thought to be important in episodic memory processing and retrieval. Work undertaken in rodents and humans, has highlighted that the existence of prior knowledge (i.e., a schema) aids the acquisition of information congruent to the pre-existing schema (Tse et al., 2007). A key example of such has been shown in rodents through an associative inference task: when rats are taught that object A will yield a reward from object B and object B will yield a reward from object C, they are able to transitively infer that object A will also result in a reward being obtainable from object C (Bunsey & Eichenbaum, 1996). Interestingly, hippocampal lesioned animals are able to learn and remember the initial object-reward associations, however, are unable to make the inference (object A to object C) compared to sham-lesioned animals. This suggests the hippocampus is crucial in the initial encoding of newly learned information and is also responsible for transferring the information into relevant schema organisations.

In terms of context-dependent memories, schemas are important in providing a framework from which relevant memories or declarative components can be retrieved. Essentially, schemas allow any encoded component to act as the context guiding decision making. For example, when a stimulus in the external environment activates a component within a schema, related components are also activated and become available for retrieval. The medial prefrontal cortex is therefore vital in managing the increased interference created by interconnected components within a schema.

Notably, the salience of the context can vary between tasks, with medial prefrontal activity modulated by factors such as the environment the animal is in, e.g., the room (Hyman et al., 2012) as well as the temporal phase of the task, such as during the inter-trial interval (Jung et al., 1998). Remarkably, medial prefrontal activity has also been shown to shift depending on *why* an animal is performing a specific action, even when the behaviours themselves are identical but performed under different contingencies (Rich & Shapiro, 2009).

The role of the medial prefrontal cortex and hippocampus during schema acquisition is believed to be both complementary and time-dependent, with newly learned information being initially encoded by the hippocampus but then over time consolidated into neocortical regions such as the medial prefrontal cortex – potentially during sleep (Sterpenich et al., 2007) – to form more long-term or remote memories (Frankland & Bontempi, 2005). This was seen in a human functional magnetic resonance imaging (fMRI) study where participants were shown and tested on the recognition of forty-location association pairs one day, one week and three months after initial learning (Takashima et al., 2006). Over time, medial prefrontal activity increased during retrieval of the association pairs and was inversely correlated with hippocampal activity. Similar results have been replicated in rats with damage to the rodent medial prefrontal cortex (specifically the anterior cingulate cortex) affecting successful retrieval of remote but not recent memories (Frankland et al., 2004).

More recently, in a study by Tompary and Davachi (2017), human participants were taught object-location associations in the form of ‘scenes’ with some objects being used more than once to create overlapping memories. The participants were then tested on recollection of which objects belonged to which location both immediately after learning and eight days later. fMRI results from the study showed greater similarity in neural activity for overlapping memories than non-overlapping memories in the hippocampus but only for remote memories (eight days post-learning). This evidence agrees with the general consensus that the hippocampus orthogonalises new information to prevent interference from previously stored memories (McClelland et al., 1995), allowing related yet distinct memories to be stored and retrieved without interference upon which the medial prefrontal cortex can then act. The data also suggests that the memories retained within the neocortex are not replicates of the initial memory but instead represent more scaffold-like formations that maintain the

overall structure of memories but lack the specific level of detail that the originals hold. These details are instead stored within the hippocampus and are retrieved upon recollection of the schema – connectivity between the anterior hippocampus and medial prefrontal cortex is greater for overlapping remote memories compared to non-overlapping recent memories (Tompary & Davachi, 2017).

1.7. Conceptual Validity and Caution in Cross-Species Comparison

While this thesis draws on a cross-species approach to examine the neural mechanisms underlying context-dependent memory, it is essential to acknowledge the conceptual and methodological challenges inherent in comparative neuroscience. Constructs such as “context,” “schema,” and “inference” are employed throughout as common descriptors of cognitive operations across rodents, marmosets and macaques. However, these terms are mainly used as helpful tools for designing experiments and interpreting results, rather than implying that different species share identical cognitive or neural processes.

Substantial anatomical and functional differences, particularly in the prefrontal cortex, complicate straightforward comparisons. For instance, the granular organisation and expanded connectivity of the primate prefrontal cortex afford levels of abstraction and executive control that are unlikely to be fully recapitulated in rodents. Conversely, rodents may rely more heavily on sensorimotor associations and habitual strategies. As such, while behaviourally similar outcomes may be observed, the underlying computations and circuits involved may differ in important ways.

As such, comparative studies must balance the translational power of shared tasks with a critical appraisal of species-specific adaptations. This thesis takes these complexities into account by interpreting results in light of known brain and behavioural differences, viewing cross-species variation not as a problem but as a useful way to understand how memory systems have evolved. Future work will benefit from explicitly testing which aspects of cognition are conserved and which emerge uniquely in more complex or linguistically enabled brains.

1.8. Conclusion

In summary, evidence from the literature points towards a bidirectional connectivity model that involves bottom-up communication between the hippocampus and medial

prefrontal cortex and subsequent top-down regulation from the medial prefrontal cortex back to the hippocampus. More specifically, the hippocampus is important in providing the medial prefrontal cortex with information regarding the current context and object stimuli with which the medial prefrontal cortex can then act upon and further direct the hippocampus as to the appropriate memory to retrieve based on which schema present within the medial prefrontal cortex was activated. At present, this is a simplified view of how the two regions interact, and as such there are still questions that remain to be answered. Work within nonhuman primates should expand this restricted knowledge base by acting as a bridge between the rodent and human studies that currently dominate the field. Cross-species comparison between all rodents, primates and humans will not only provide an insight into the evolutionary and neurobiological origins of how we successfully encode and process context-dependent memories but will also prove important when deciphering how humans utilise language and semantics to enrich their contextual understanding.

Chapter 2: Temporal Context-Guided Memory Sequencing in Lister Hooded Rats

2.1. Introduction

How we remember our day-to-day experiences often involves recalling sequences of events tied to specific contexts (Davachi & DuBrow, 2015; Schapiro et al., 2013). For example, when thinking about your commute to work (by bicycle or by car), you may recall the events leading up to it, such as eating breakfast, as well as what happened when you arrived (e.g., greeting colleagues or heading to the coffee machine). These events, though individual, are linked together to collectively form a distinct memory (Baldassano et al., 2017), which is critical for guiding actions and predicting outcomes (Robin & Moscovitch, 2017). By reconstructing sequences, humans can navigate complex environments, anticipate outcomes, and resolve ambiguities when events overlap (Dunbar, 2003; Kurby & Zacks, 2008; Bar, 2009).

Human episodic memory excels at recalling sequences spanning multiple locations, a skill likely tied to evolutionary pressures to navigate and forage effectively (Milton, 1981; Sherry & Schacter, 1987). This ability to encode and retrieve overlapping sequences across contexts underpins how episodic memory supports goal-directed behaviour. It allows individuals to manage ambiguous or overlapping information, a challenge inherent to navigating dynamic and unpredictable environments (Brown et al., 2010). To model these features, researchers have extensively explored rodent learning paradigms, focusing on how these animals form associations between environments and stimuli.

While decades of research have demonstrated that rodents can form robust associations between environments and stimuli – such as in context-fear paradigms where rats associate a specific environment with an aversive event (Fanselow & Poulos, 2005; Herry & Johansen, 2014; Tovote et al., 2015; Maren et al., 1997, 2013) – fewer studies have explored their ability to encode and recall temporal sequences that overlap across environments. Research indicates that rats can disambiguate overlapping sequences (Agster et al., 2002; Wood et al., 2000) or contexts suggesting their ability to form schemas – neural frameworks that allow the integration of new memories and the modification of existing ones (Kesteren et al., 2012). It remains

unclear, however, whether these schemas can support the integration of sequences across multiple contexts, a hallmark of human episodic memory.

Resolving ambiguity in overlapping sequences is integral for human episodic memory, for daily experiences often involve shared information regarding people, places, or events between memory episodes (Schacter et al., 2011; Jacques et al., 2013). And yet, for most individuals, these overlapping memories are effectively managed, and everyday occurrences are remembered when needed. To do this, humans use context to guide retrieval, a reliance mirrored in rodent models (Brown et al., 2014; Roberts & Clarke, 2019), which have demonstrated similar capabilities under controlled conditions. Navawongse & Eichenbaum (2013) and Place et al. (2016) demonstrated that rats can disambiguate overlapping objects by using contextual cues. These studies reveal the importance of context in resolving ambiguity but primarily address spatial associations. They do not incorporate the temporal complexity inherent in human episodic memory, where events must be linked and recalled in sequence across contexts.

Building on this work, a temporal component was introduced to examine how rats learn object-context associations that unfold in sequential order. By requiring rats to choose objects in an order dependent on which context was approached first, this method introduces greater complexity and ambiguity, resembling human memory more closely. Drawing on prior human studies (Tompary & Davachi, 2017; Reeders et al., 2021), which tested the ability to recall several objects present within and across contexts, we hypothesised that sequences confined to a single context would be learned and recalled more easily than those spanning multiple contexts due to increased conflict from overlapping information.

2.2. Methods

2.2.1. Subjects

Nine male Lister Hooded rats (*Rattus norvegicus*) supplied from Charles River, UK, were initially recruited for the study. Due to difficulties during the habituation phase, one rat did not meet the criteria to proceed to the training and testing stages. Consequently, the final sample comprised eight rats that completed the full experimental protocol. All rats were housed in cages of three, in a room on a 12-hour light-dark cycle from 07:00 to 19:00 hr, with temperature (20 ± 1 °C) and humidity (55 ± 10 %) kept stable. The cages in which the animals were housed (each measured, L: 56 x W: 38 x H: 22 cm, supplied by RC2F, NKP isotec., UK) included a red translucent tunnel that would later be used as a method of transportation. Experimentation occurred during the light phase with food and water freely available throughout. Animals were not euthanised as part of the experiments. All procedures undertaken were in accordance with the guidelines of the UK Animals (Scientific Procedures) Act of 1986 and approved by Durham University AWERB and the Home Office (procedure licence number: PP8877096). Reporting follows the recommendations in the ARRIVE guidelines.

2.2.2. Apparatus

The maze (Figure 2.1A) consisted of two square contexts (L: 50 x W: 50 x H: 50 cm) joined to a corridor that spanned the length of the two contexts (L: 100 x W: 25 x H: 50 cm) adjacent to one another. Each context was distinct with individually patterned walls and removable inserts (L: 50 x W: 50 cm) changing the texture of the floor. One insert was made of grey Lego and the other from black rubber matting. The entire maze (L: 100 x W: 75 x H: 50 cm) was made of 10 mm thick PVC foam sheet and painted using spray paint. Entrances to each context (L: 15 x W: 12 cm) were blocked by a moveable door attached to the top of the maze by fishing string. The floors of each context included four circular wells (diameter: 4 cm x depth: 2 cm) spaced equidistantly from the four walls of the context and each other (24 cm, Figure 2.1A-B). The wells contained stainless steel cups (diameter: 4 cm x depth: 2 cm) that were removable. Atop of each well was one of four objects (of which there were three copies of each, Figure 2.1C). All objects were distinct in colour, size, and shape. The maze itself was in a room with no obvious visual landmarks available to the animals inside

the maze. The testing room was dimly lit (diffuse white light from a lamp; 100 W) and had white noise playing in the background to prevent disturbance from noises outside the room. Disinfectant wipes (Clinell universal wipes, GAMA Healthcare Ltd., UK) were used to clean all objects, floor inserts and the maze. This consistently occurred after every trial.

2.2.3. Habituation Procedure

2.2.3.1. Handling

Rats were habituated to the handler and testing room prior to experimentation. Habituation began with three days of handling in the housing room with each cage being handled for 15 min in total per day. During this time, each animal within the cage was picked up via the red translucent tube; this was to reduce the need for unnecessary contact and promote a less stressful and anxious environment (Gouveia & Hurst, 2019). The process was repeated to last the length of the session with all animals being handled an equal number of times. Once acclimated to both the handler and the handling procedure, each cage of three rats was handled in the testing room for 10 min a day for six consecutive days.

2.2.3.2. Maze Habituation

To introduce the rats to the maze, one of the three housing cages was brought to the testing room and all rats inside were allowed to explore the entire maze as a group over the course of 30 min. Excretion levels were monitored to help identify if any of the rats were particularly anxious about being in the maze and food pellets (45 mg LabTab MLab, Indiana, USA), the reward used later in the experiment, were generously scattered to promote exploration of all areas. After one day of group habituation, the animals were introduced to the maze individually and allowed to explore the entirety of the maze uninterrupted. The rats were picked up using the red tube as indicated previously and placed in the corridor of the maze with their heads facing the two doors. Similarly, the red tube was also implemented when removing the rats from the maze, during the tenth minute of the session, again with movement only occurring in the corridor. The procedure of moving the animals via the red tube was kept consistent throughout the entirety of the experiment. Individual maze habituation was carried out over two days and was followed by two days of handling in the testing room to further

solidify the importance of movement both in and out of the red transport tube. One animal was excluded at this stage due to persistent difficulties habituating to the testing environment, reducing the final sample to eight rats.

2.2.3.3. Shuttling

Rats were trained to shuttle between the two contexts of the maze, with each animal participating in a 10 min session that was repeated over four days, within which they were guided to enter and leave one of the contexts by the use of food pellets. The doors of the maze were controlled by a string attached to a pulley that the handler could lift and lower at their own discretion. During a typical shuttling session, a pellet was placed in the centre of both contexts and a door to a context was opened allowing the animal to enter and retrieve the pellet. Upon entering the context, the door was lowered with the animal being kept inside. A pellet was then placed in the centre of the corridor before the door to the context was opened once more and the animal was allowed to leave and retrieve the newly placed pellet. This was repeated several times during the session with the context the animal was required to shuttle to and from being pseudo-randomly selected. Each animal was permitted to progress to the next level of habituation once 95 % of the shuttle runs they performed within a 10 min session were under one minute in duration.

2.2.3.4. Object Habituation

After successfully shuttling between contexts, the four objects the animals would encounter during the experiment were introduced individually. This involved each object being presented to the rat in the location that they would be kept in during testing. As seen in Figure 2.1B-C, object A (“purple bear”) was placed in the top left corner and object B (“orange pump”) was placed in the top right corner. In the Blue context, object C (“pink watering-can”) was placed in the bottom left corner and object D (“green shaker”) was placed in the bottom right corner. At first, a pellet was placed directly adjacent to the object to promote maximum interaction but then once the animal was familiar with the object, they were taught to retrieve the pellet from the well underneath the object. This was achieved by placing the correct object next to the well with a pellet inside and following each subsequent interaction, repositioning the object so that it incrementally covered the well. With the first few interactions, the animal could still see the pellet but had to push the object away in order to retrieve the pellet.

By the time the object completely covered the well, the animal knew where the pellet was and how to obtain it. Over the course of four days, each object was presented individually beginning with object A on the first day and ending with object D on the fourth day. Each animal was required to shuttle in and out of the context as previously described, with progression to the training stage only occurring once 95 % of the runs were under one minute long. Again, each habituation session lasted 10 min.

2.2.4. Training Procedure

Once the animals had learned to associate a food pellet with each of the four objects, training began.

2.2.4.1. One object in one context

To start, the rats were exposed to the two object pairs associated with each context (i.e., objects A and B in the Yellow context and objects C and D in the Blue context). The aim was to teach the animal, that upon first entry, either object A or C was to be chosen and then upon second entry, the correct object was either B or D. To ensure the animals learned the correct order, only one object was present in a given context at any one time. As such, each rat was taught to shuttle in and out of the same context, first collecting a pellet from under the object present on the left (object A if in the Yellow context or C in the Blue context), and then upon returning to the same context, from underneath the object on the right (object B in the Yellow context and object D in the Blue context). Manually opening and closing the doors to direct the animals into specific contexts gave the experimenter an opportunity to rebait the objects or change which objects were present between visits. Each rat was allowed to explore the other wells and the context itself, however, if during a run, the animal took longer than five minutes to retrieve a pellet, the run was aborted. In error trials, the rat was removed from the maze before being allowed to retrieve any food and placed in a time-out cage for two minutes and the trial was aborted. All runs were counterbalanced, and each rat undertook six runs per day. This stage of training lasted a week with each rat completing 36 runs in total.

2.2.4.2. One object in two contexts

Next, the rats were exposed to the two object pairs associated across contexts (i.e., objects A and D going from Yellow to Blue context and objects C and B going from

Blue to Yellow context). Much like the first training step, this was to teach the rats that sequence could be split across contexts and that entry into a context denoted the sequence order. Again, only the correct object was present during an entry, and each rat had to shuttle between the two separate contexts, first collecting a pellet from the object present on the left in the first context, and then upon entering the second context, from underneath the object on the right. The experimenter manually opened and closed the doors to control the animal's transition between the contexts. All wells and the context were freely available to be explored, but if the animal took longer than five minutes to obtain the pellet, the run was aborted with the animal being transferred to the time-out cage for two minutes.

2.2.4.3. Two objects in one context

By this point, all rats had learned that the object on the left yielded a reward upon first entry of a context, and then upon re-entry it was the object on the right that now yielded a reward. To ensure the animals were not simply reliant on only one object being present from which to collect their reward, both objects were presented at once, requiring the rats to decide which one was correct. To aid their learning, the correct object was baited, and the incorrect object was not. If the rats were incorrect in their choosing, they were removed from the maze by the handler and placed in the time-out cage for two minutes – the run being subsequently aborted.

2.2.5. Testing Procedure

2.2.5.1. Single context phase: temporal order sequence in one context

For the first stage of testing, the rats undertook a version of the 'Two objects in one context' training step (Section 2.2.4.3), this time with both wells baited to prevent the rats using the odour of the pellet reward as a cue to the location of the correct response. Manually opening and closing the doors gave the experimenter the opportunity to control the rats' transitions between contexts. Each rat performed six trials every day, and all trials were counterbalanced so that testing of sequence learning in either the Yellow or Blue context was equal. An example trial involved a door to one of the contexts being opened by the experimenter and the animal having to push over the object they deemed to be correct. If the context was Yellow, this was object A (Figure 2.2A). The animal then shuttled out of the context to the corridor, and

the experimenter rebaited both objects, before the animal was allowed to re-enter and select the second object (again if Yellow, the correct choice upon re-entry was object B). Alternatively, if a trial involved the Blue context, the correct order of objects was object C followed by object D upon re-entry (Figure 2.2B). If the rats were incorrect in their choosing, they were removed from the maze by the handler before being able to retrieve any food and placed in the time-out cage for two minutes with the trial being aborted. During all error trials, runs were aborted, and the animal was removed from the maze for two minutes. To proceed to the next stage of testing animals were required to achieve a total of more than 10 correct trials out of 12 trials across two days (threshold performance), corresponding to a performance significantly above chance (binomial test, $p = 0.019$, one-tailed).

2.2.5.2. Two context phase: temporal order sequence across two different contexts

Once successfully above threshold, the rats progressed to the second testing stage whereby they had to remember the object pairs across contexts. This was similar to the second training step where the rats were taught to shuttle between contexts (Section 2.2.4.2), except now, two baited objects were present in each context to remove any bias caused by odour from the reward. As such, each rat needed to decide which of the two objects available was the correct choice depending on which context was entered. For example, a trial that transitioned from Yellow to Blue context (Figure 2.2C) involved the animal shuttling from the Yellow context – retrieving the pellet from under object A, out of a possible choice of object A or B – to the Blue context where it retrieved the pellet from under object D (after visibly seeing objects C and D together). Conversely, if the animal was shown the Blue context at the start of the trial, the animal needed to shuttle from the Blue context to the Yellow context, selecting object C in the Blue context followed by object B in the Yellow context (Figure 2.2D).

Initially, all wells were baited; however, once rats reached the threshold – defined as a total of at least 10 correct trials out of 12 across two consecutive days – they were transitioned to a version in which only the correct object(s) were baited. Between trials, the interior of the apparatus was cleaned, along with objects and floor inserts, to prevent contamination from odour cues. This modification was introduced to streamline behavioural management and reduce the need for experimenter intervention for incorrect choices. In the previous part of the phase, the experimenter

intervened to prevent animals from retrieving rewards before an incorrect selection; although this approach was effective, the change to baiting only the correct object(s) allowed for more consistent control over error correction while minimising potential disruption to the animals. Critically, this adjustment provided sufficient time for the experimenter to intervene before the animal could self-correct by subsequently switching to the rewarded object, thereby ensuring clearer trial outcomes. Additionally, by baiting only the correct object(s), it ensured that animals could not receive a reward for an incorrect choice in instances where the experimenter was unable to intervene in time. Rats that again met the performance criterion under these conditions proceeded to the final testing stage.

2.2.5.3. Combined temporal order and context sequences

The last testing stage utilised the rats' movement between the two contexts and required them to select out of the four objects, which one was correct for the context they entered. All four objects were the same across the two contexts both in identity and location. Only the correct object was baited, and the rats had to perform at threshold level in order to progress to the final stage. The final testing stage was identical to that of the penultimate stage (Section 2.2.5.2) except that the animal now had control over which context they entered and which order in a given trial. This meant the rat could utilise any of the four logical sequences (Figure 2.2E-H) they had learned throughout the experiment so long as a correct decision was made upon each entry, otherwise they were removed from the maze without reward and placed in the time-out cage for two minutes with the trial being subsequently aborted.

2.2.6. Behavioural analyses

Error ratios, defined as the total number of a specific error type divided by the total number of trials performed by each rat (Figure 2.3A), were calculated to compare performance across individuals during both the single context and two context phases of the experiment.

To evaluate the impact of context and error type on error ratios, two linear mixed effects models were fitted. Random intercepts for each rat were included to account for individual variability in baseline error rates. Fixed effects were tested using a type-III hypothesis test, ensuring that the contribution of predictors was assessed after

accounting for shared variance with other factors. Results were considered significant at $p < .05$.

Prior to conducting comparisons, the normality of the data was assessed using the Shapiro-Wilk test applied to error ratios, and outliers were identified based on the interquartile range (k = 1.5). No deviations from normality were detected, and no outliers required removal. Post-hoc comparisons between groups were performed where relevant, and Cohen's d was calculated to quantify effect sizes for differences between means.

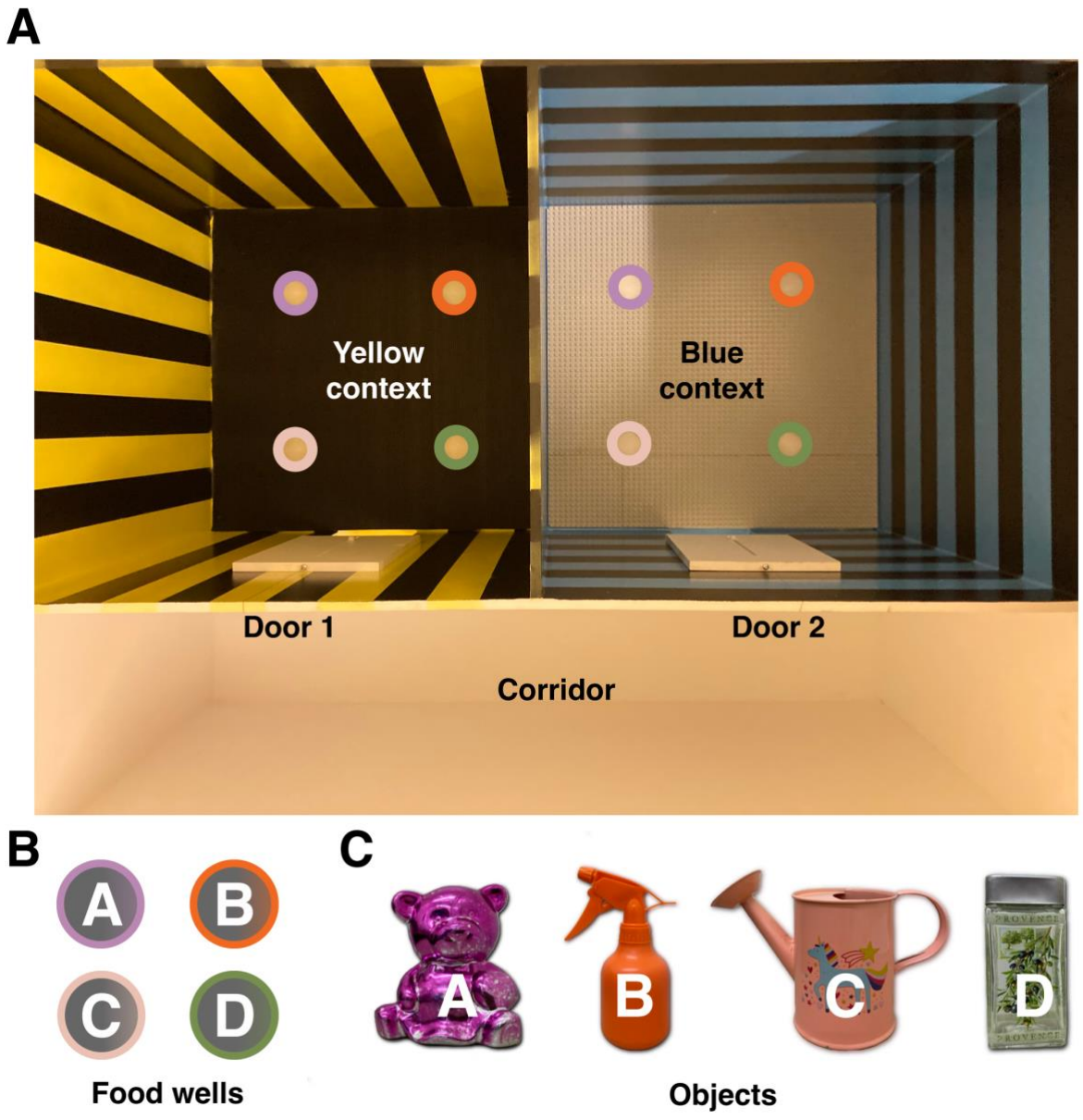


Figure 2.1. Aerial view of the maze, food wells and objects.

A) *An aerial view of the maze, with two contexts – 'Yellow context' on the left and 'Blue context' on the right.* ***B)*** *Shows four wells within the maze.* ***C)*** *Displays the arrangement of four objects placed consistently above the wells in each context: 'A' over the top left well, 'B' over the top right well, 'C' over the bottom left well and 'D' over the bottom right well.*

2.3. Results

2.3.1. Phase 1: Single context object pair learning

The first phase of testing evaluated whether the eight starting rats could recall the correct temporal order of objects when presented with just two objects per context (Figure 2.2A-B). Testing was conducted in a maze comprising two distinct square contexts connected by a corridor. Each context contained four food wells with two specific objects positioned over food wells located in the floor. In the Yellow context, object A was positioned in the top-left corner and object B was placed in the top-right corner (Figure 2.2A). Similarly, in the Blue context object C was positioned in the bottom-left corner and object D in the bottom-right corner (Figure 2.2B). This meant from each rat's perspective, objects C and D were closer to the entrance to the context than objects A and B.

During this phase, rats were exposed to one context per trial. In a given trial, the rats were required to complete a two-item sequence. Upon initial entry into the context, the rats were required to choose from the two objects (e.g. object A in the Yellow context) and, shuttling out and re-entering, to then choose the second object of the pair (e.g., object B). Object choice involved a rat pushing over an object to reveal the well underneath where a pellet could be retrieved as a reward. To eliminate odour-based cues, both wells under the objects were baited with food pellets, ensuring that selection was only guided by learned behaviour. Trials were counterbalanced across contexts to account for any potential bias, and incorrect choices resulted in removal of the rat to a 'time-out' cage for two minutes, with the trial being aborted. Each rat completed six trials per day, with success defined as achieving a threshold of 10 correct responses out of 12 trials over two consecutive days (p = 0.019, binomial test, one-tailed).

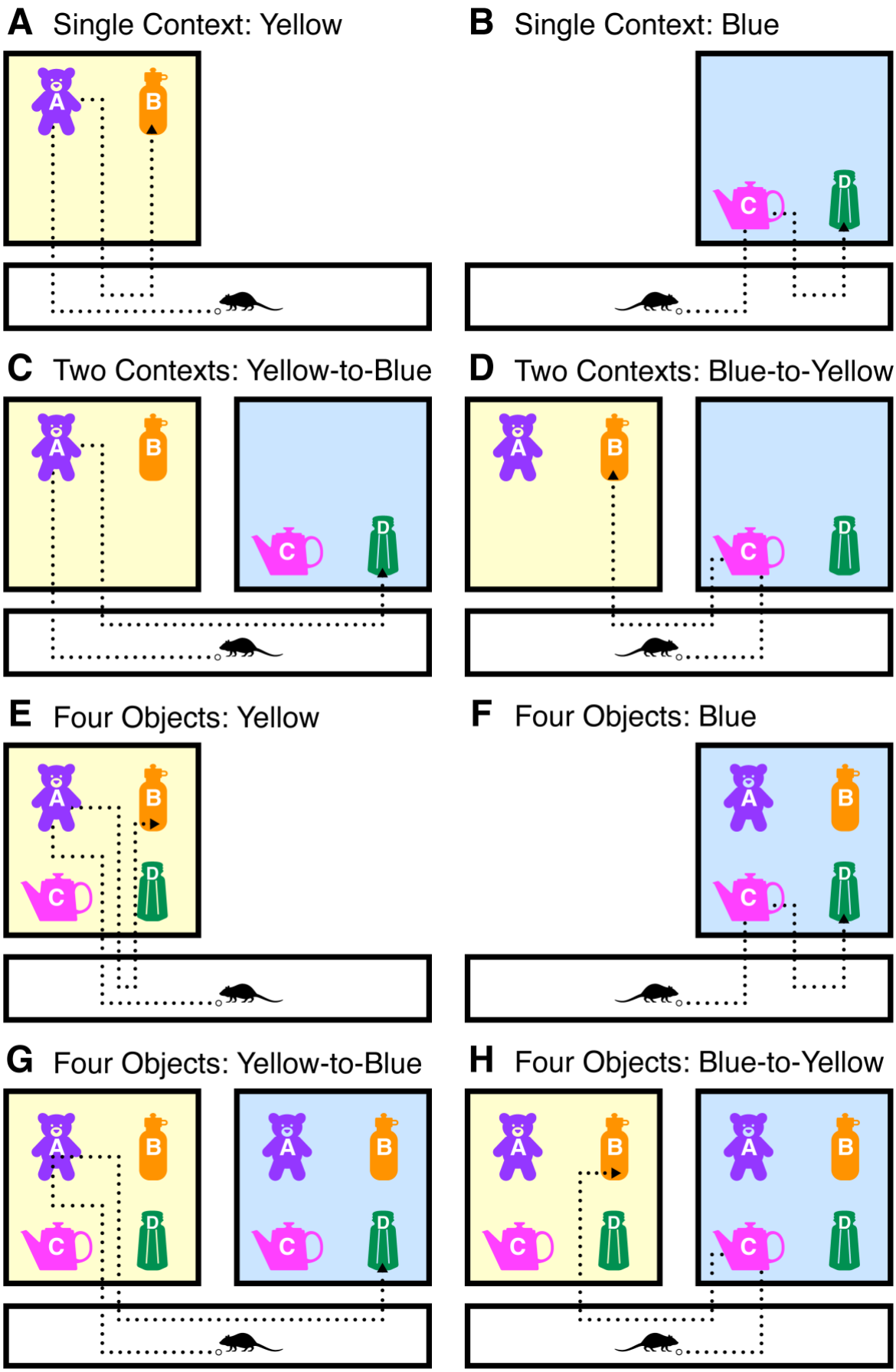

A Single Context: Yellow
B Single Context: Blue
C Two Contexts: Yellow-to-Blue
D Two Contexts: Blue-to-Yellow
E Four Objects: Yellow
F Four Objects: Blue
G Four Objects: Yellow-to-Blue
H Four Objects: Blue-to-Yellow
A
B
C
D

Figure 2.2. Schematic representation of testing stages and object sequences across single and two contexts.

A) and B) *The correct object order for sequences in the single context phase (Phase 1), with* ***A)*** *the correct ordering in the Yellow context and* ***(B)*** *in the Blue context.* ***C) and D)*** *The correct object order in the two context phase (Phase 2), with* ***C)*** *representing the transition from Yellow-to-Blue context, and* ***D)*** *the transition from Blue-to-Yellow context.* ***E) to H)*** *The four possible configurations for testing, with all objects (A, B, C, D) present in context during the final phase (Phase 3). These configurations reflect the combinations of single context trials* ***E) and F)*** *and two context trials* ***G) and H)****.*

Out of the eight animals that were tested, six successfully met criterion (Figure 2.3A). Across the full cohort – including those that did not reach criterion – the average number of days taken to reach or attempt to reach criterion was 13.9 days (SD: 10.8; Figure 2.3B). The two rats that failed to reach criterion underwent the full experimental time, completing all 31 days without meeting threshold performance.

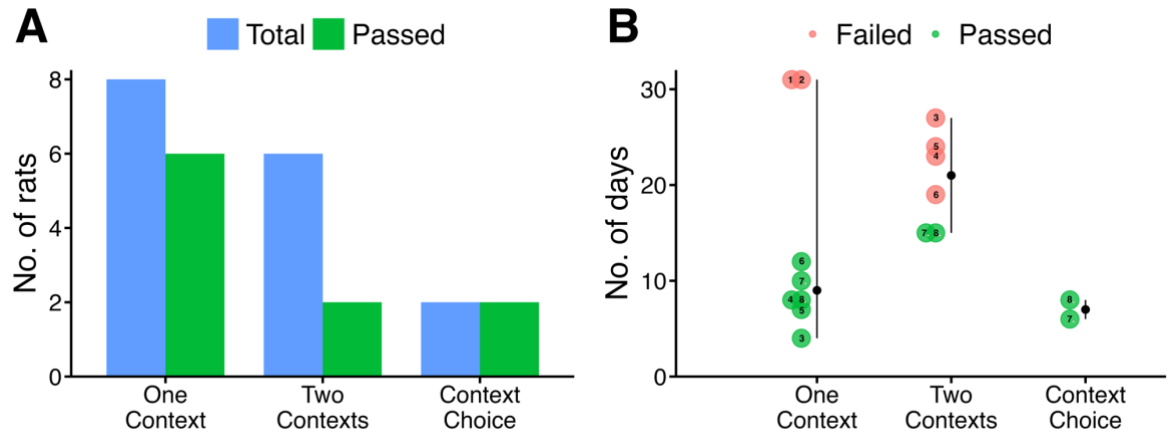


Figure 2.3. Number of rats and days needed to pass each testing stage.

A) *The total number of rats tested at each phase (blue bars) and the number that successfully met the predefined criterion (green bars). The criterion for success was achieving 10 correct responses out of 12 trials (83 % correct) over two consecutive days (p = 0.019, binomial test).* ***B)*** *The median number of days spent on each testing phase, with rats that successfully completed the phase (green) and those that did not reach criterion (red) identified. Testing was capped at a maximum of 31 days for all rats, regardless of their progression. ID of each rat is provided with numbers 1 to 8.*

2.3.2. Phase 2: Bridging object order across contexts proves challenging

Rats that reached criterion in the single context phase (n = 6) progressed to the next testing phase involving an object pair that spanned two contexts. Each trial began in one context, and rats were required to choose the correct object before moving to the other context to complete the trial. Figure 2.2C-D display the two possible sequences the animals could encounter. For example, transitioning from the Yellow context to the Blue context required choosing object A first in the Yellow context, followed by object D in the Blue context (Figure 2.2C). The reverse transition from Blue context to Yellow context required choosing object C first in the Blue context followed by object B in the Yellow context (Figure 2.2D). Learning the sequences that spanned across the two contexts proved difficult for the rats, with only two of six rats successfully reaching criterion performance (Figure 2.3A). Across the full cohort, including those that failed to reach criterion, the average number of testing days was 20.5 (± SD: 4.97; Figure 2.3B). Notably, the two rats that reached criterion did so in the fewest number of days, each completing in 15 days (Figure 2.3B).

2.3.3. Phase 3: Adapting to change in context – all four objects

In the final phase, the two successful rats from the second phase were tested on their ability to freely select a context to enter and complete the correct object choice accordingly. Both contexts now contained all four objects (objects A, B, C, and D), increasing the complexity by introducing potential distractors and testing if the presence of incongruent objects interfered with the rats' ability to remember the correct temporal order (Figure 2.2E-H). Rats were allowed to choose their starting context, with the subsequent object order determined by the chosen context.

Both rats successfully reached criterion within an average of 7 days (mean: 7.00 ± SD: 1.41, Figure 2.3A-B), completing this phase considerably faster than earlier stages. The behavioural data from this phase were not formally analysed, as the primary aim was to assess whether the rats were capable of flexibly selecting and applying learned sequences under more complex conditions. A breakdown of the trial types attempted, along with the outcome frequencies for each rat, is provided in Table 2.1. These results demonstrate the rats' ability to integrate prior learning, adapt to changing demands, and overcome interference from distractor objects.

Table 2.1. Frequencies and proportions of responses across four possible trial types for each rat in Phase 3.

Rat ID	Trial Type	Correct *n*	Half-Correct *n*	Incorrect *n*	Total Trials *n (%)*
7	Yellow (YY)	0	0	0	0 (0)
	Blue (BB)	0	0	0	0 (0)
	Yellow-Blue (YB)	13	3	2	18 (50)
	Blue-Yellow (BY)	12	4	2	18 (50)
8	Yellow (YY)	1	0	0	1 (2)
	Blue (BB)	8	0	0	8 (19)
	Yellow-Blue (YB)	15	3	2	20 (47)
	Blue-Yellow (BY)	9	5	0	14 (33)

Note: Five trials were excluded for Rat 8 as they were started but were incorrect (4 in Blue and 1 in Yellow).

Across the three experimental phases, the findings highlight how rats acquire and apply temporal and spatial order memory under increasingly complex conditions. In single contexts, most rats successfully learned the temporal sequence of object choices, showcasing their ability to associate temporal order with specific spatial environments. Bridging temporal sequences across two distinct contexts, however, proved significantly more challenging, with only two out of six rats reaching criterion. This suggests that integrating spatial transitions with temporal order memory imposes a substantial cognitive load, as it requires combining multiple memory components. Despite this complexity, two successful rats demonstrated, through freely choosing context-sequences, their ability to learn and flexibly retrieve sequences, indicating a capacity for integrating and applying this type of memory.

2.3.4. Does context change between trials influence performance at the group level?

Secondary analyses for the entire cohort of rats were conducted to explore factors contributing to the rats' difficulty in learning object pairs across contexts. These cohort error analyses aimed to identify how prior trials, single or multiple contexts and objects affected error rates.

Error ratios were calculated for each rat by dividing the number of errors by the total number of trials attempted for each testing phase (Figure 2.4A). This allowed for direct comparisons for group performance at each phase.

To examine the impact of context change, trials were categorised based on whether the context of the preceding trial had changed or remained the same (Figure 2.4B). A trial was classified as "context changed" if it occurred in a different context from the previous trial (e.g., switching from Blue to Yellow) and "context same" if both trials occurred in the same context (e.g., both trials in Blue). This categorisation enabled an evaluation of whether performance on a given trial was influenced by residual contextual information carried over from the previous trial.

To explore the factors contributing to increased errors in the cohort of rats, trials were analysed using a linear mixed-effects model. Trials were ordered sequentially for each rat across all sessions, comparing the context of the current trial with that of the previous trial (Figure 2.4B). In the two context phase, where two contexts were presented per trial, performance was compared on the last context of a trial with the starting context of the next trial (illustrated in Figure 2.4C). This approach was used to determine whether rats adapted to the context changes (indicating low error rates) or whether certain factors – such as biases toward prior actions or previous object locations – contributed to increased errors.

All rats that participated in the single context phase (n = 8) and the two context phase (n = 6) were included in the analyses. Additionally, errors were categorised as '1st Object' and '2nd Object' errors, corresponding to the object within the object pair on which the rat failed (Figure 2.4D-E). This classification was applied to both single context and two context trials to further examine how stable versus changing contexts within and between trials influenced performance.

For the single context phase, a linear mixed-effects model with error ratio as the dependent variable was applied. Error type (1st or 2nd Object errors) and context change (changed or same) were included as fixed effects, with a random intercept for individual rats to account for variability. An interaction term (error type × context change) was also included to assess whether context change influenced error rates differently depending on error type.

The analysis revealed a significant main effect of context change, with higher error rates when the context changed (mean: 0.14 ± SD: 0.10) compared to when it remained the same (mean: 0.06 ± SD: 0.05; F (1, 21) = 15.7, p < .001, Figure 2.5A). This suggests that rats struggled to update contextual information between trials and instead tended to persist with previous trial actions when faced with a contextual change.

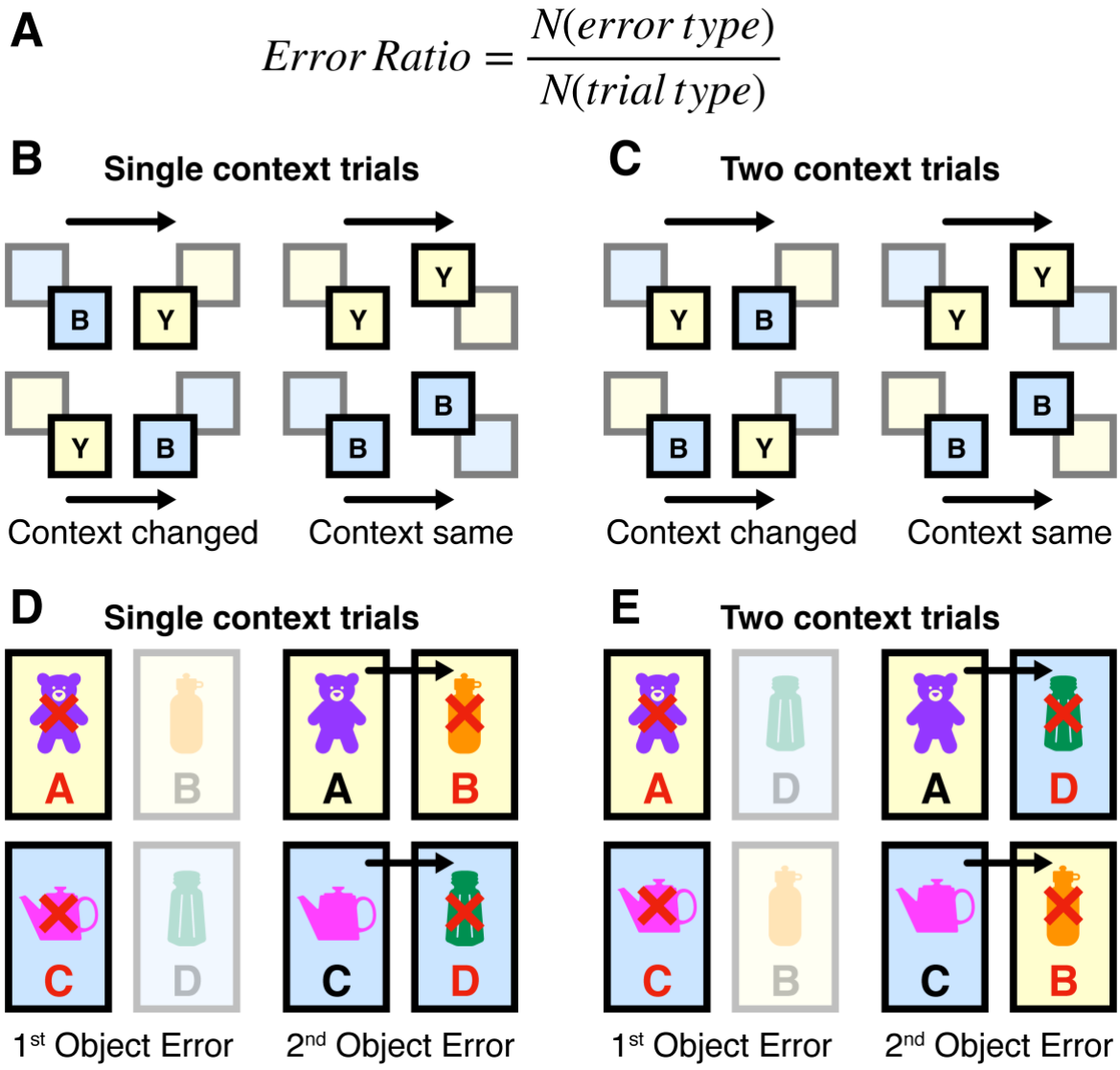


Figure 2.4. Examples of error ratio for each error and trial type.

A) *Error ratios were calculated based on the number of errors divided by the total number of trials attempted.* ***B) and C)*** *Illustrate the categorisation of error types in single context and two context trials, respectively, focusing on context transitions between trials. Specifically, context change was defined by comparing the context in which the preceding trial ended to the context where the subsequent trial began. For example, if the first trial ended in the Yellow context and the next trial began in the Blue context, this transition was classified as a 'changed' context, even if the trial sequence itself remained the same (i.e., going from a Blue-Yellow trial to a Blue-Yellow trial).* ***D) and E)*** *Depict error types in single context and two context trials, respectively, focusing on object selection. Here, errors were further differentiated into '1st Object' and '2nd Object' errors, based on which object within the sequence the rat choose incorrectly.*

Post-hoc analysis revealed that when the context remained the same, errors for the first and second object were low and did not significantly differ (t (21) = 1.17, p = 0.255, d = 0.585, Figure 2.5C). However, when the context changed from that present on the prior trial, rats were significantly more likely make an error on the first object (mean: 0.20 ± SD: 0.07) than on the second object (mean: 0.09 ± SD: 0.08; t (21) = 3.62, p < .001, d = 1.81, Figure 2.5C).

This pattern is consistent with the rats repeating their previous trial action rather than adapting to the change in context. Specifically, when the context changed, rats made significantly more errors choosing the first object, indicating they were heading directly to the second object instead. Given that the second object was always positioned on the right side, this behaviour is consistent with perseveration to the last rewarded location or adherence to a procedural learning rule (i.e., choosing the location of the object in the prior context).

However, this pattern was different when the context remained the same. In these cases, error rates were lower and there was no significant difference in first and second object selection errors, implying that rats were more likely to correctly choose the first object and effectively 'restart' the new object pair order.

For two context trials, a similar linear mixed-effects model was utilised, but the pattern of results was reversed from that of the single context trials considered in the previous section. Here, error rates were significantly *lower* when the context changed (mean: 0.07 ± SD: 0.04) than when it remained the same (mean: 0.17 ± 0.08; t (15) = - 4.32, p < .001, d = -1.73, Figure 2.5B). This finding suggests that, under two context conditions, errors were more likely to occur when the context was stable between trials. Unlike in the single context phase, where errors increased following a context change, in two context trials, errors were more frequent when the context remained the same, suggesting a shift in strategy relative to the single context data.

The rats' behavioural errors on the two context trials suggest that context changes influenced their decision-making process. Specifically, when the context remained the same between trials, rats tended to initiate the trial correctly, showing a statistical trend in being more likely to choose the first rather than the second object (t (15) = 2.06, p = 0.058, d = 1.19, type-III hypothesis test, Figure 2.5D). These observations suggest

that the rats that progressed to the two context phases were better able to identify the start of a new trial but struggled to adjust their response for the second object when the context changed within the current trial.

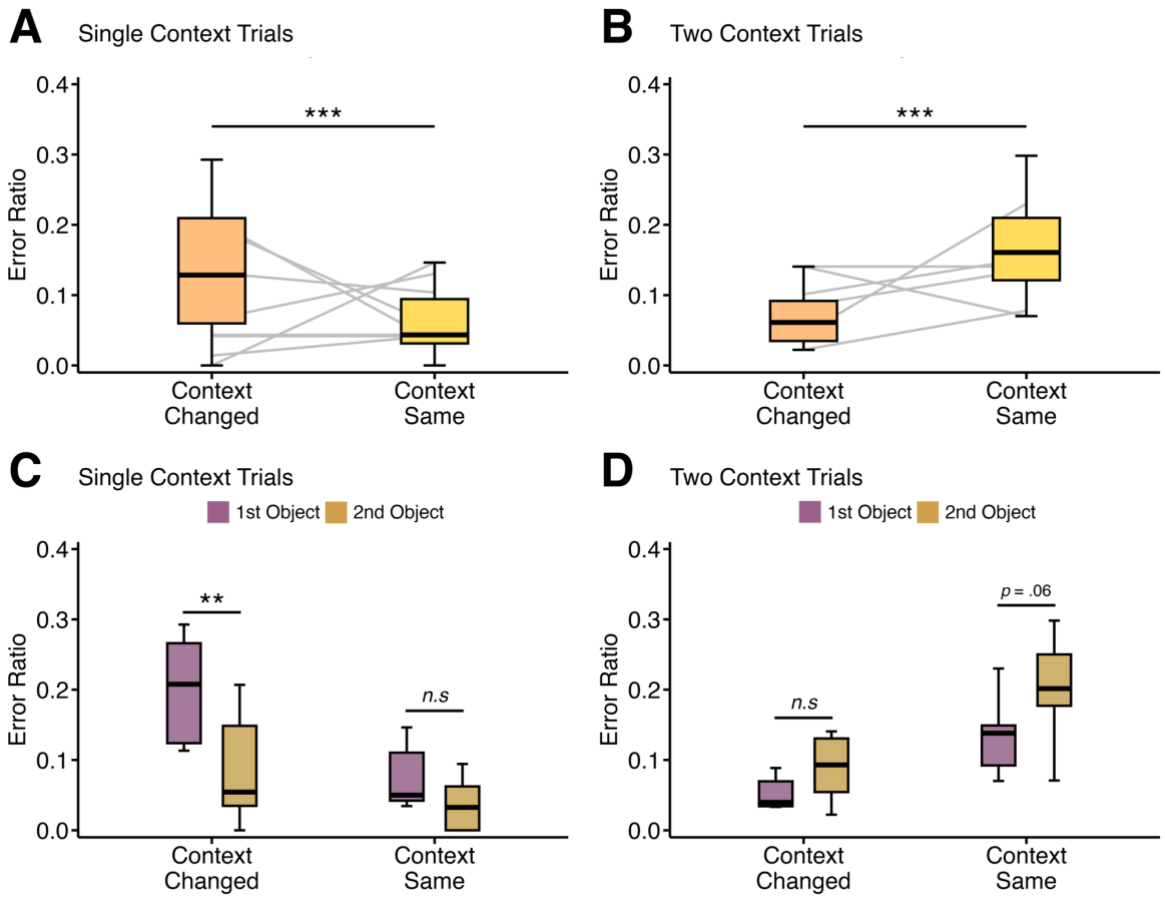


Figure 2.5. Error ratios when context changed and remained the same between trials as ordered by individual rat.

Error ratios based on whether the preceding trial changed ('Context Changed') or remained the same ('Context Same') when trials were ordered by individual rat. ***A)*** *Error ratios for single context trials, showing significantly higher errors when the context changed compared to when it remained the same ($p < .001$).* ***B)*** *Error ratios for two context sequence trials, showing significantly lower errors when the context changed compared to when it remained the same ($p < .001$).* ***C)*** *Error ratios for '1st Object' and '2nd Object' errors in single context trials. Rats made significantly more "1st Object" errors when the context changed ($p < .01$) but not when the context remained the same.* ***D)*** *Error ratios for '1st Object' and '2nd Object' errors in two context trials. No significant differences were observed, though a trend suggested increased '2nd Object' errors when the context remained the same ($p = .06$). Significance is indicated by *** ($p < .001$), * ($p < .05$) or n.s ($p > .05$).*

2.3.5. Is there a bias towards certain contexts?

To examine how specific contexts influenced error types, error rates in individual contexts (Yellow and Blue) across single context and two context trials were analysed. This analysis aimed to determine whether rats' performance was affected by the specific context they were in and whether their behavioural strategies differed within and between contexts. Data from all rats were included (n = 8) for single context trials or the six rats (n = 6) that progressed to two context trials, with errors grouped by individual rats in each context.

For the single context phase, a linear mixed-effects model was used, with error ratio as the dependent variable and error type (1st or 2nd Object errors) and context colour (Yellow or Blue) as fixed effects. A random intercept for rat accounted for individual variability. The model also included an interaction term (error type × context colour) to determine whether the effect of context colour on error rates differed by error type.

The model revealed no main effect of context (F (1, 21) = 4.30, p = 0.051) nor for the interaction between context and error type (F (1, 21) = 0.995, p = 0.330), however, did reveal a significant main effect of error type (F (1, 21) = 9.87, p = 0.005).

In single context trials, there was a statistical trend toward higher error rates in the Yellow context compared to the Blue context (t (21) = 2.07, p = 0.051, d = 0.733, Figure 2.6A), suggesting that the Yellow context trended towards posing greater difficulty for the rats.

In the Yellow context, rats made significantly more first object errors (mean: 0.35 ± SD: 0.18) compared to second object errors (mean: 0.15 ± SD: 0.18, t (21) = 2.92, p = 0.008, d = 1.46, Figure 2.6C). No significant difference in error type was observed in the Blue context (t (21) = 1.52, p = 0.144, d = 0.758, Figure 2.6C), although the mean differences appear to be in a similar direction.

Similarly, for the two context phase, another linear mixed-effects model was used, with error ratio again as the dependent variable and error type (1st or 2nd Object errors) and context colour (Yellow-to-Blue or Blue-to-Yellow) as fixed effects. A random intercept for rat accounted for individual variability. The model also included an interaction term

(error type × context colour) to determine whether the effect of context colour on error rates differed by error type.

The model revealed no main effect of context (F (1, 15) = 2.84, p = 0.113) nor for error type (F (1, 21) = 3.13, p = 0.0.097), however, did reveal a significant main effect for the interaction between context and error type (F (1, 15) = 18.95, p < .001).

In two context trials, error rates did not differ under the two contexts (t (15) = 1.29, p = 0.113, d = 1.69, Figure 2.6B). However, when analysing error types, rats showed greater difficulty in the Yellow context, particularly when it was the final context of a trial. Second object errors significantly increased in Blue-to-Yellow trials (t (15) = 4.33, p < 0.001, d = 2.50, Figure 2.6D), whereas the opposite pattern was a statistical trend for the reverse transition (Yellow-to-Blue; t (15) = 1.83, p = 0.088, d = 1.05, Figure 2.6D).

This suggests that while rats could initiate object pair choices in the Yellow context, they struggled to complete them in the Yellow context, which might explain why only two rats were able to advance through all testing phases. The overall pattern of results is consistent with context sensitivity particularly for the two context conditions, which unlike the single context phase, are difficult to explain by a procedural learning rule (last object location or left-right alternation strategy). These observations also suggest that the rats did not appear to be strictly following a procedural rule – such as a left-right alternation strategy, switching sides for reward retrieval upon each entry into any context.

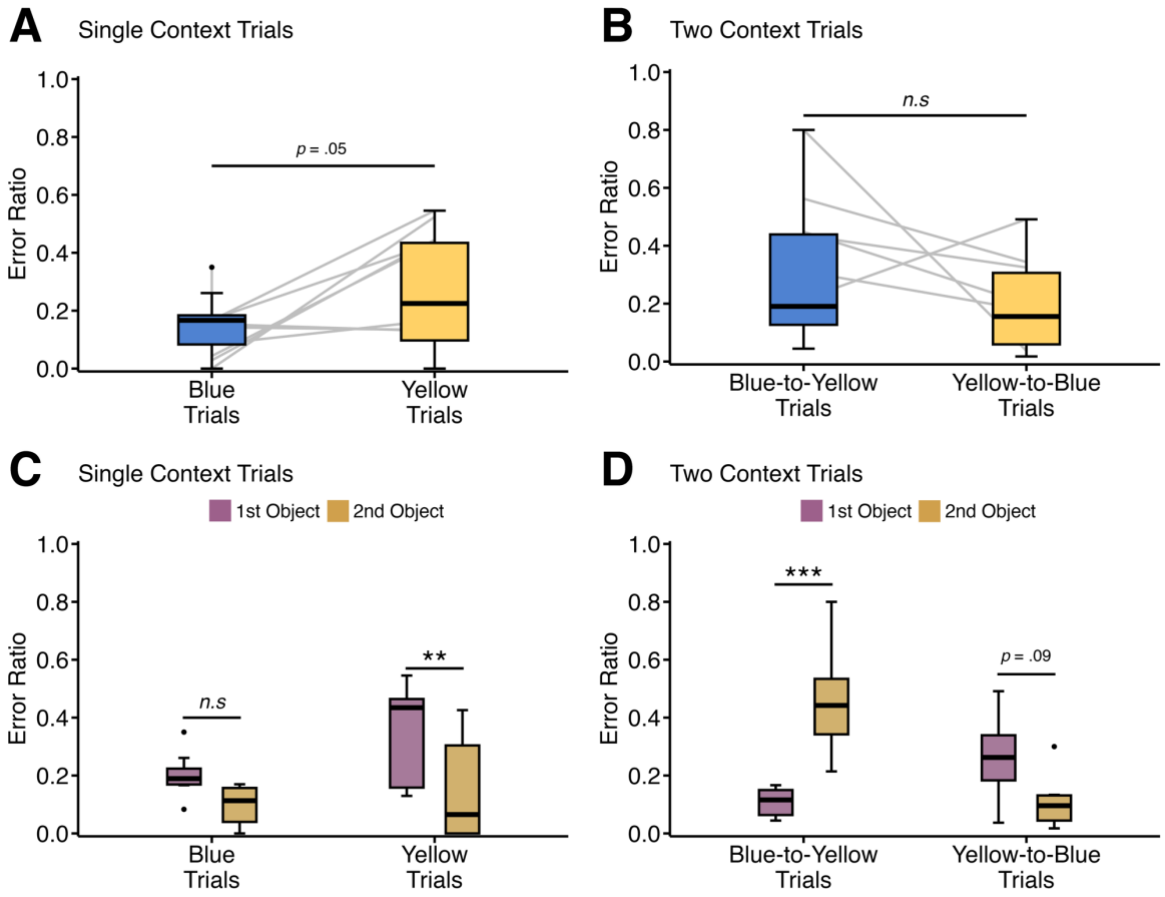


Figure 2.6. Error ratios across Blue and Yellow trials in single and two context trials ordered by individual rat.

Error ratios and error type comparisons for Blue and Yellow trials in single context and two context phases. ***A)*** *No significant difference in overall error ratios between Blue and Yellow trials during the single context phase (p = .051).* ***B)*** *During single context trials, rats made significantly more '1st Object' errors than '2nd Object' errors in Yellow trials (p = .008), but no significant difference was observed in Blue trials (p = .144).* ***C)*** *In the two context phase, no overall difference in error ratios was found between Blue-to-Yellow and Yellow-to-Blue transitions (p = .113).* ***D)*** *A significant increase in '2nd Object' errors was observed in Blue-to-Yellow trials compared to '1st Object' errors (p < .001), while no significant difference was found in Yellow-to-Blue trials (p = .088). Significance is indicated by *** (p < .001), * (p < .05) or n.s (p > .05).*

2.4. Discussion

Being able to remove ambiguity from sequences of overlapping events is a component of episodic memory that humans excel at, with the contextual environment of a memory thought to be the main driving force behind the ability to recall the correct order of events (Clarke et al., 2022). Despite the ubiquitous nature of this type of memory in humans, modelling and testing of such an occurrence is difficult, particularly in nonhuman animals. Previous research has demonstrated that rats are adept at associating aversive stimuli with a contextual environment and are capable of deciphering object-context associations even when objects are present in more than one context (Navawongse & Eichenbaum, 2013; Place et al., 2016). A caveat of such research is the use of singular objects and stimuli that are dependent on limited contexts, thus lacking the characteristic ambiguity associated with multiple sequences of events across multiple contexts as is seen in human episodic memory. As such, in the current study, we assessed the ability of eight Lister-hooded rats in remembering two sequences of objects whose temporal order depended on the order in which the contexts were visited. The task implemented the use of a two context maze; within each context which were up to four objects whose identity and location were kept the same for both contexts. Animals had to rely on the context they were in and the context prior to determine the correct order of objects.

Rats demonstrated a capability in learning temporal order within a single context but displayed a difficulty in adjusting previously obtained knowledge to accommodate for the order of known objects across the two known contexts. Out of the eight rats tested, six were successful in reaching threshold performance when the two-object sequence remained within a single context. When the same six were tested on completing a sequence that involved a change in context mid-trial, only two rats were successful in learning this more complicated sequence. Following further analysis, it was identified that the presence of the rat in the preceding trial had no subsequent effect on the following rat's performance, suggesting that accuracy of performance was reflected in each rat's individual experience and was not influenced by others.

As well, for both types of sequence, it was shown that rats made the most errors when the last context of a trial differed to the first context of the following trial, further highlighting the effect each rat's own experience had on their subsequent

performance. Contextual analysis also highlighted that rats were more prone to errors choosing the first object in the Yellow context, potentially due to increased caution or environmental differences, but not in the Blue context. Notably, when transitioning from Blue to Yellow, rats had more trouble choosing the second object, implying challenges in adapting strategies, potentially due to increased object distance and interference in the Yellow context. Overall, the data indicate that rats often repeated previous decisions and faced context-specific challenges that influenced their error patterns and choice strategies.

In essence, the way memories are recalled for the single context sequences is reliant on first, the physical context, i.e., the colour pattern of the walls and floor, and then secondly, the temporal order, i.e., whether or not this is the first or second entry into the physical space (Figure 2.7A). These memories can be referred to as static as their lack of ambiguity allows them to be kept distinct and separate from one another. For example, rats can quickly discern that objects A and B are only relevant in the Yellow context and objects C and D are only important in the Blue context. Once these initial associations have been learned, order of the objects can be appropriately identified with relative ease. As a result, recall of the correct object pair relies heavily on the surrounding context or environment.

Research into the hippocampal system of rodents is well founded and the hippocampus has been shown repeatedly to be important for spatial and temporal order memory of objects (O'Keefe, 1976; Lisman, 1999; Fortin et al., 2002; Forwood et al., 2005). It is, therefore, not a broad leap to suggest that memory of single context sequences is predominantly hippocampal dependent; the appropriate rule can be logically determined following natural movement through each environment. In this instance, the rat enters a context and makes a choice, and upon subsequent entry to the same context moments later, makes the opposite choice after evaluating the object order.

The difficulty in discerning the correct order arises when the sequences overlap across the two contexts as the objects are no longer only associated with just one context but with both, providing ambiguity through an increased number of possible choices. As a result, memory of the required order is no longer purely hippocampal dependent as comparison of multiple overlapping memories is required. In addition to the

hippocampus, involvement of more frontal regions is necessary to enable this effective comparison (Badre & Nee, 2018; Avigan et al., 2020). Previous research by Eichenbaum and colleagues posits that appropriate memory retrieval is guided by the medial prefrontal cortex, particularly in rodents (Preston & Eichenbaum, 2013). In the aforementioned context-guided object association task (Navawongse & Eichenbaum, 2013), rats needed to discern using their surrounding context from which two objects they would receive a reward, despite witnessing the same two objects in different contexts. Through a series of experiments, it was shown that initial contextual information is fed forward to the medial prefrontal cortex from the hippocampus and upon object selection, memory suppression is fed back to the hippocampus to bias the correct response (Rajasethupathy et al., 2015). In the case of the work undertaken by Eichenbaum and the present study, prefrontal engagement is only necessary, therefore, when ambiguity arises, and the animal is required to dissociate overlapping or 'competing' memories.

In considering how rats might overcome this difficulty and recall temporal sequences across two contexts, a possible method – and one that prevents the need to remember all object associations – is for the rats to categorise not by physical context as was the case in the previous step, but instead in terms of context entry. For example, if the rat ignores the lengthy object associations such as 'object A precedes object B and object D' and instead considers object A and object C in the category of 'objects rewarded upon first entry' and objects B and D as 'objects rewarded upon second entry', then remembering the correct sequence is much simpler. This categorisation approach aligns with findings from Crystal & Smith (2014), who indicate that rats can form bound representations of episodes involving multiple features and contexts that are resistant to interference and can persist over long retention intervals. They suggest that similar mechanisms facilitate the handling of overlapping episodic memories by integrating features such as "what", "where", "source," and "context". Consequently, in the present study, when the rat enters the Yellow context, instead of recalling the numerous sequences to determine the correct object, if the 'first entry' category is recalled, the rat can systematically work out which object is relevant based on the surrounding context (the correct answer being object A). This method can be carried forward when the rat then transitions to the Blue context, for if the 'second entry' category is brought

to mind, the rat can decipher that only object D is relevant and will be the object to yield a reward (Figure 2.7B).

From the data collected, it is clear that the second testing phase proved particularly difficult for the rats to learn. Importantly, however, the rats that were successful in learning these two context sequences showed similar performance in all four possible sequence types (Figure 2.2E-H) when given the freedom to choose the order of the contexts in the final phase of the experiment. Not only does this reinforce the fact that they had successfully learned the more complicated two context sequences, but it also demonstrates that the original single context sequences were not 'overwritten' in the learning process. In fact, to be able to reliably and consistently 'choose' whichever sequences they wanted, the rats needed to have obtained a form of flexible schema of both rules and sequences that they could utilise when necessary and that could be adapted on the availability of where they could travel (Gilboa & Marlatte, 2017). Hence, the model described in Figure 2.7B accommodates for this and removes the rigidity of the previously learned rules shown in Figure 2.7A for the single context sequences.

In all testing phases described, categorisation can aid memory recall, however, only in testing phase two and three, where sequences spanned across two contexts or had the possibility to span across more than one distinct context, was categorisation necessary. Considering, therefore, very few rats succeeded in learning the latter two phases, poses the question as to how easily can rats learn to categorise events? McKenzie et al. (2014) replicated the object-context study conducted by Navawongse & Eichenbaum (2013) with the addition of electrophysiology of cells in CA1 and CA3 regions of the hippocampus, to suggest that context-dependent information is recalled in a hierarchical order; the first of which being the context of the environment, followed by spatial position of the objects, valence of each object before finally the item identity of the rewarded object. This schema is suggested to be how the rats were able to identify the correct object even when the objects were identical across different contexts. In many ways, this is what is described in the model outlined in Figure 2.7A, the main difference being that rats needed to complete a two object sequence unlike in the experiment conducted by McKenzie et al. (2014). The schema changes, as such, for the second phase where it is suggested that context entry precedes spatial context during the decision-making process (Figure 2.7B). The hierarchical property is

still very much prominent; however, the most pertinent information has now replaced pole position.

One potential limitation of the study is the order of training and testing, which may have influenced the rats' ability to adapt to the task. Rats were initially trained on single context sequences before being tested on the same phase. Had the two context sequences been tested first, the rats might have adapted more quickly to the single context temporal sequences, as they would have been able to more easily adjust their schema. Furthermore, the design of the testing environments may have introduced a behavioural bias, with rats displaying improved performance for trials in the Blue context compared to the Yellow context. This effect was evident in secondary analyses examining the impact of context on error type; for both single and two context trials, the Yellow context was associated with more errors.

This effect may not have been solely driven by the physical characteristics of the contexts, but rather by the rats' potential reliance on the experimenter's position as a contextual cue. The experimenter stood on the left side of the Yellow context (nearest to object A) and on the right side of the Blue context (nearest to object D) during testing, in order to remove the rat if an error was made. If the rats were using the experimenter's position as a cue, then we would expect trials transitioning from Yellow to Blue (object A to D sequence) to have fewer errors, as the experimenter's position would guide the animal to the correct object. This pattern was observed, with rats making fewer errors during the transition from Yellow to Blue. However, further analysis revealed that rats made more errors choosing the second object when transitioning from Blue to Yellow (incorrectly selecting the object in Yellow) and more errors selecting the first object when transitioning from Yellow to Blue (also incorrectly selecting the object in Yellow). While this could reflect a bias based on the experimenter's position, it is more likely a contextual bias. As to which aspect of the context created this bias is unclear, but it might potentially involve the proximity of objects to the entry point (with Yellow objects being further away from the entry point than in the Blue context) or environmental differences, such as the brightness of the contexts (though not directly tested), which may have contributed to these error patterns.

In an attempt to bridge the animal and human literature and provide a more comparable model of the human behaviour surrounding contextualisation of sequences of events, the current study advances our understanding of the memory capabilities of rodents and demonstrates that rats can learn sequences of objects that are contained within a context far more easily than sequences that span multiple contexts. It is important to note that the generalisability of our findings is limited by the small number of rats that completed all phases of the experiment. Further studies with larger samples sizes are necessary to confirm these results and assess their broader applicability. Future experiments may also be able to shed light on the neurobiological mechanisms that underlie this distinction through *in vivo* manipulation of brain pathways that are integral to consolidatory mechanisms involved in creating and maintaining mental schemas.

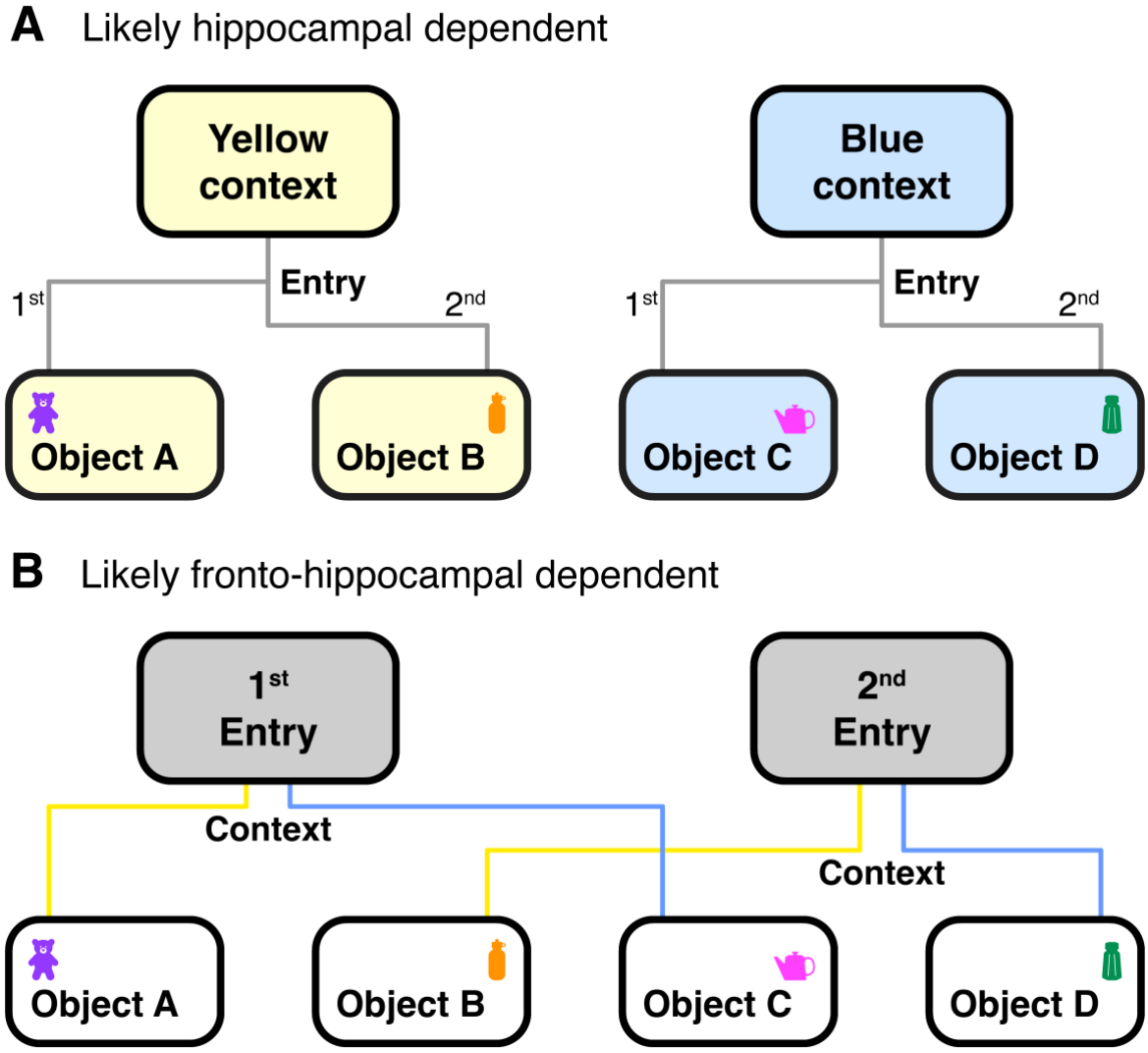


Figure 2.7. Schematic of potential memory pathways for single and two context sequences.

A) *Shows two distinct memory pathways for single context sequences, either in the Yellow or Blue context, where physical context precedes the order in which objects are remembered.* ***B)*** *Illustrates overlapping memories for two context sequences, where memories are categorised by the temporal order of context entry rather than the physical context, i.e., the Yellow or Blue context.*

Chapter 3: The Capability of Common Marmosets in Learning Temporal Context-Guided Sequences

3.1. Introduction

In recent years, the use of common marmosets (*Callithrix jacchus*) in biomedical research has gained considerable traction, driven primarily by the development of transgenic marmoset models for studying neurological diseases such as Alzheimer's and Parkinson's (Okano et al., 2012; Rizzo et al., 2023). Marmosets are also becoming increasingly favoured in animal research due to their high reproductive rates, relatively low costs, and ease of maintenance compared to other nonhuman primate models (Tardif et al., 2003; Abbott et al., 2003). In addition to their neurobiological relevance, their small size, allows for naturalistic, freely moving studies of cognitive behaviour that would otherwise be more challenging to conduct with larger species such as rhesus macaques (Meisner et al., 2024). As such, this combination of factors has led to a 1.5-fold increase in publications including the term "marmoset" over the past decade (Figure 3.1A), with the United Kingdom being the second highest contributor following the United States (Figure 3.1B, Scopus, 2024).

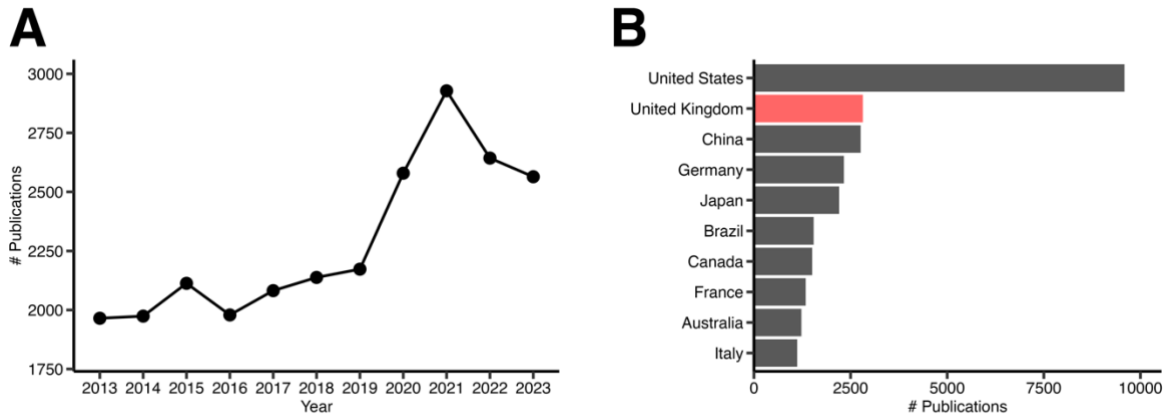


Figure 3.1. Trends in research publications involving the term "marmoset" from 2013 – 2023.

A) *The number of publications per year from 2013 to 2023 to include the search term "marmoset".* ***B)*** *The distribution of publications by country, focusing on the top ten contributors.*

Marmosets offer an opportunity to bridge the cognitive research gap between rodents and larger primates as their cognitive and behavioural characteristics position them as strong models for comparative cognition. Their behavioural characteristics, in particular, make them a suitable and valuable model for studying human-like cognition than more commonly used murine models, such as rats and mice. Unlike murine models, which are primarily solitary, nocturnal, and rely heavily on olfaction, marmosets are diurnal, visually attentive, and exhibit complex social structures mirroring humans (Miller et al., 2016). Additionally, marmosets perform well in tasks that require working memory and cognitive abilities (Marx, 2016), positioning them between simpler rodent models and more sophisticated nonhuman primate models, such as rhesus macaques that are often sought after for studies of higher cognition.

Alongside their behavioural similarities, marmosets also serve as a natural translational bridge between rodent and macaque models from a neurobiological perspective. Belonging to the class of New World monkeys, marmosets diverged from humans approximately 45 million years ago (Preuss, 2019), placing them evolutionarily between rodents, which diverged ~ 90 million years ago, and Old World monkeys, such as macaques, which diverged ~ 35 million years ago. In terms of absolute brain size, marmosets have brains that are five times larger than those of rats but ten times smaller than those of macaques (Herculano-Houzel, 2009). Remarkably, the organisation of key brain areas, such as sensory, motor and association cortices, is similar across all three species (Van Essen et al., 2019). This shared brain structure facilitates investigation of similar cognitive processes and mechanisms across species.

Furthermore, the prefrontal cortex of marmosets occupies an intermediary position between that of rodents and larger primates (including macaques and humans). While rats have a prefrontal cortex composed predominantly of agranular regions, such as the prelimbic, infralimbic and anterior cingulate cortices (Heukelum et al., 2020; Bizon et al., 2012), marmosets possess both agranular and granular areas, similar to the canonical prefrontal model in macaques, albeit with fewer subdivisions (Burman & Rosa, 2009). The presence of granular areas is considered a specialisation of primates (Preuss & Wise, 2022) and is thought to be absent in rats (Burman et al., 2006). This neural architecture is involved in various cognitive processes, including

decision-making and social behavior (Birrell & Brown, 2000), further emphasising the significance of these areas as being pivotal in modelling higher cognitive functions.

Research into the true extent of marmoset cognitive ability has been somewhat overshadowed by research focusing either on their social behaviour or phenotypic display of neurodegeneration. This is primarily due to their quick maturation, reaching adulthood within a year of birth (Perez-Cruz & Rodriguez-Callejas, 2023). Marmosets that reach 8 years old are considered aged, exhibiting various signs of neurological impairment, including reduced hippocampal neurogenesis and beta-amyloid deposition in the cerebral cortex (Lacreuse et al., 2014), making them suitable models for research into memory decline in aged individuals (Castro & Girard, 2021).

Sequential learning and the ability to understand the temporal order of events have been demonstrated in marmosets and other members of the *Callitrichidae* family. In marmoset groups, social status plays a significant role in determining each individual's access to food and breeding opportunities. Breeding pairs are typically codominant, sharing the highest status, while the hierarchy among nonbreeding animals is determined by age, with younger individuals holding a lower rank in the group (Digby, 1995). Therefore, it is imperative for marmosets to understand and learn the ordinal associations and social hierarchy of individuals within their social group. This naturally hierarchical behaviour suggests marmosets may possess strong abilities in tasks that require sequencing and ordered learning.

To test this ability beyond the ethological perspective of social rank, Koba et al. (2012) trained five marmosets to touch four graphical objects in a specific order (i.e., object A, then B, then C, then D). Their capacity to plan sequences was assessed by shuffling the positions of the objects on the screen after the first object was selected correctly. The marmosets successfully learned the sequence when object positions remained static within a trial and could complete the sequence accurately regardless of the order in which the objects were presented. Although accuracy did not significantly differ between shuffled and static conditions, the response time for selecting the second object in the sequence was significantly longer in the shuffled position.

Additionally, the marmosets were tested on their ability to learn relative positioning by presenting only two items from the sequence at a time (e.g., selecting B followed by C, when objects B and C were randomly displayed on the screen). All marmosets

successfully identified the correct order of the stimulus pairs, though response latency significantly increased as the number of missing objects within the sequence grew. This highlights the marmosets' capability to form an internal linear representation of stimuli and infer their order transitively, a trait observed and shared with other nonhuman primates and rodents (Terrace, 1993; Bunsey & Eichenbaum, 1996).

Studies conducted both in the wild and in captivity also demonstrate that marmosets can retain spatial and temporal information over long periods. For example, marmosets are able to locate and retrieve food from a previously explored location, even after a significant delay between exposure and retrieval, or when the location was only visited once (Abreu et al., 2020). This suggests that marmosets have robust spatial memory and an ability to integrate both spatial and temporal information across time. Similarly, in a laboratory setting, marmosets have shown proficiency in tasks that require visual working memory and discrimination learning. Nakamura et al. (2018) reported that marmosets could retain visual information for up to 16 seconds and their study highlighted that while initial learning required many trials, subsequent learning of novel stimuli was significantly quicker, indicating that marmosets can build upon prior knowledge to enhance their learning efficiency.

While marmosets have shown proficiency in learning fixed sequences and spatial locations, adapting sequences to varying contexts represents a more advanced aspect of memory and decision-making. This flexibility is a hallmark of episodic memory, which depends on the capacity to understand the temporal and contextual boundaries of events (Ross & Easton, 2022). Context plays a crucial role in guiding behavior across different environments, allowing animals to remember sequences across multiple events or that occur in varying situations (Robertson et al., 2015). In primates, this ability is crucial for complex decision-making and adapting to dynamic environments (including food availability over increased distances).

Little is known about the ability of marmosets to adapt to temporal context-dependent sequences, a cognitive function closely tied to the prefrontal cortex. Rodent studies have provided insights into this area, implicating regions such as the medial prefrontal cortex, in tasks involving context-dependent learning and memory. As marmosets possess a more granular and differentiated prefrontal architecture, which aligns more closely with that of higher-order primates, investigating how marmosets process

context-dependent temporal sequences provides an opportunity to explore how granular prefrontal regions contribute to this type of memory. As such, this study specifically aims to assess marmosets' capacity to learn such sequences and to examine how alternating between contexts impacts their understanding of overlapping sequences of objects. By comparing these findings with the rodent study conducted in Chapter 2, this research seeks to uncover how prefrontal specialisations influence episodic-like memory across species and whether marmoset adaptability aligns more closely with that of macaques or differs fundamentally from rodent models.

3.2. Methods

3.2.1. Subjects

Five common marmosets were tested (Figure 3.2A) and consisted of three males (TT: 9 years old, NC: 1 year 8 months and ML: 4 years old) and two females (GG: 8 years old and MT: 4 years old). GG, TT and NC (Cohort 1) were housed together in a family group, and ML and MT (Cohort 2) were housed as a pair by themselves. The home-units where the animals were housed measured 2.25 m tall, and, occupied a floor area of 1.26 m^2 and had a total volume of 2.5 m^3.

All animals resided in a colony comprised of several home-units, housing 14 marmosets in total. The colony had a 12-hour light-dark cycle from 07:00 to 19:00, with both temperature (23 – 28 °C) and humidity (40 – 70 %) kept stable. All animals tested were not fluid or food restricted and were fed a Teklad New World Primate diet (5S48, Envigo; 25 grams per two animals, soaked in warm water) in the morning and a cafeteria style diet consisting of mixed fruit, proteins and carbohydrates in the afternoon.

The animals were obtained from a breeding colony at the Defence Science and Technology Laboratory (DSTL, Porton Down, Salisbury, UK). All procedures undertaken were in accordance with the guidelines of the UK Animals (Scientific Procedures) Act of 1986. In addition, the project was approved by Newcastle University's Animal Welfare and Ethical Review Body (AWERB).

3.2.2. Apparatus

The maze (Figure 3.2B-C) consisted of two plastic boxes (Really Useful Box, London, UK) measuring internally (L: 37 cm x W: 31 cm x D: 28 cm) and externally (L: 48 cm x W: 39 cm x D: 31 cm) and joined by a PVC pipe. The maze was positioned in front of the home-unit so that the lids of the boxes were parallel with the bars of the cage (Figure 3.2C). Entry to the maze was via a circular hole cut into the lid of one of the boxes. As the lids were removable, entry to each box could be achieved by swapping the lids.

Each box acted as a distinct context and was appropriately coloured to match the rat and macaque tasks discussed in the other chapters. The box on the experimenter's right-hand side was painted yellow with black stripes that ran perpendicular to the lid

and base of the box, and the box on the left was painted blue with black stripes that ran parallel to the lid and the base of the box (Figure 3.2C). From the marmoset's perspective, when positioned in front of the home-unit, the Yellow context was on the left and the Blue context on the right, consistent again with both the rat (and macaque) experiments.

Four wooden target objects (Popetpop, UK, Figure 3.2D) were evenly spaced and attached with zip-ties to the side of box the box facing the home-unit (Figure 3.2B). Each object was a similar colour and size but had a distinct shape. Directly adjacent to the objects, a small circular hole (diameter: 1 cm) was cut into the box. This was to allow the animal to receive a reward upon choosing the correct object. Rewards typically consisted of a piece of marshmallow (Sweets and Treats Wholesale, UK).

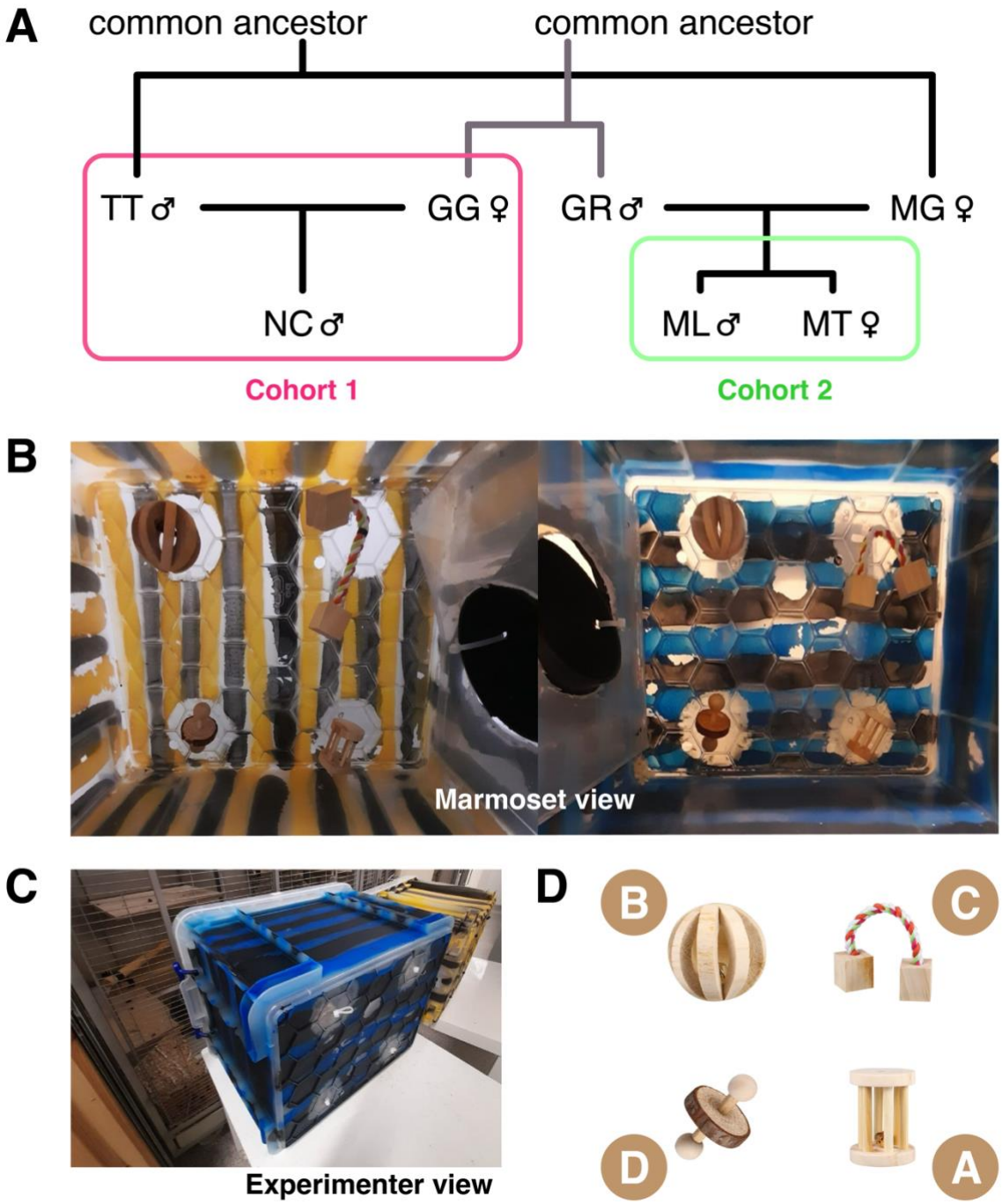


Figure 3.2. Marmoset cohort and experimental setup.

A) *Genealogical representation of the five marmosets used in the experiment. The two cohorts represent distinct family lineages with a common ancestor.* ***B)*** *Marmoset view of the maze.* ***C)*** *Experimenter view of the maze from outside the home-unit.* ***D)*** *The four wooden target objects used in the task, with their associated letter. The spatial arrangement pf the objects depicts their actual arrangement in the maze as the marmosets would have witnessed.*

3.2.3. Habituation Procedure

To acclimatise the animals to the maze, the maze was left outside the home-unit for 10 minutes with no direct access, but animals could touch the maze through the bars if they desired. Next, the maze was attached to the home-unit and all animals in a given cohort were allowed 10 minutes of uninterrupted access via the Yellow context, and then 10 minutes via the Blue context. Food was added to both boxes and the tunnel to promote further and thorough exploration. Acclimatisation was deemed complete once all animals exhibited relaxed behaviours and showed interest in both boxes.

3.2.4. Training and Testing Procedure

For both cohorts, during both training and testing, each animal was separated from the social group in which they were housed via positive reinforcement (e.g., food reward). This was achieved via positioning the maze at the bottom of the home-unit and carefully inserting dividers above the maze to gently guide the desired animal into the 'testing area' below, whilst allowing the remaining members of the group to occupy the space above. If signs of distress were seen (including excessive vocalising, agitated movement or attempts to get back to the social group) the session was aborted, and the animal was returned. In addition, a trial was deemed to start when the animal entered the box and was considered complete when more than one-third of the animal's body, excluding the tail, had exited the doorway.

The training and testing procedure mirrored that explained in the rat (and macaque) chapter(s) with slight changes made to accommodate for the different species. Additionally, subtle adjustments were made to the training and testing parameters throughout and are reported accordingly. To further ease comprehension, reporting of procedures has been split by cohort with Cohort 1 being the first group of marmosets trained and tested and with the maze being positioned on the bottom-half of the home-unit (Figure 3.2C). Cohort 2 were tested second, and had the maze positioned on the top-half of the home-unit for half of the experimental period. This is explained in further detail later on.

3.2.4.1. Cohort 1

For Cohort 1, training began by first teaching each animal a sequence of objects within a single context through use of food and auditory luring (Figure 3.3A-B). This involved

enticing the animals towards one target using a piece of marshmallow and then redirecting them towards the next object in a similar manner once they had interacted with the first target. A bridge (an auditory click produced by the experimenter already established from previous husbandry training) was used to pair the correct action and reward. If the context was yellow, the sequence ran from object A to B (Figure 3.3A), and if the context was blue, the correct sequence involved object C followed by object D (Figure 3.3B). For Cohort 1, each animal performed 10 trials per session and to promote learning through exploration without imposing error penalties, animals moved on to the next training phase after fully completing 17 out of 20 trials across two consecutive sessions. A fully completed trial involved interacting with the two correct objects in their required order and independently exiting the maze.

In the first phase of training, animals were rewarded for approaching the correct objects by use of luring. The second phase of training involved luring towards the correct objects and rewarding for grasping the target objects. The third phase of training allowed the animals to perform as many trials as they desired within 10 minutes. The experimenter kept their hands in a neutral position out of sight of the marmosets, and only rewarded after an object was clearly grasped for longer than two seconds. If an incorrect object was grasped the trial was aborted. If an animal struggled with the sequence order of objects, occasionally the animal was lured to the correct object to help solidify learning. Progression to the next phase occurred once an animal consistently performed at about or above 75 % correct in non-lured trials over multiple sessions on both types of trials (Yellow and Blue contexts).

The animals were then tested on the single context trials (Figure 3.3A-B), using the same approach as previously stated, except this time with no luring to the correct object if confused during a trial. The threshold to pass this phase was classified as having achieved 30 out of 40 trials correct across two consecutive testing sessions. Due to limited access to the colony for welfare reasons, training and testing did not occur every day of the week and there were periods in which the marmosets went without being trained or tested.

Following testing of single context sequences, animals were trained in sequences that spanned across two contexts (Figure 3.3C-3D). Much like the training for single context sequences, animals were lured to the relevant objects only when necessary

and there were no correct or incorrect trials. As access was only via one context, the context in which the animal could access the maze was switched every session. In this phase, within each session, only a single type of two-context sequence was trained; that is, all trials in one session were either Yellow-Blue (object A in Yellow followed by object D in Blue, Figure 3.3C) or Blue-Yellow (object C in Blue followed by object B in Yellow, Figure 3.3D). The context sequence direction alternated between sessions but was consistent across all trials within a session. Each animal was trained on 20 trials per session and needed to have fully completed 30 out of 40 trials across two sessions to progress.

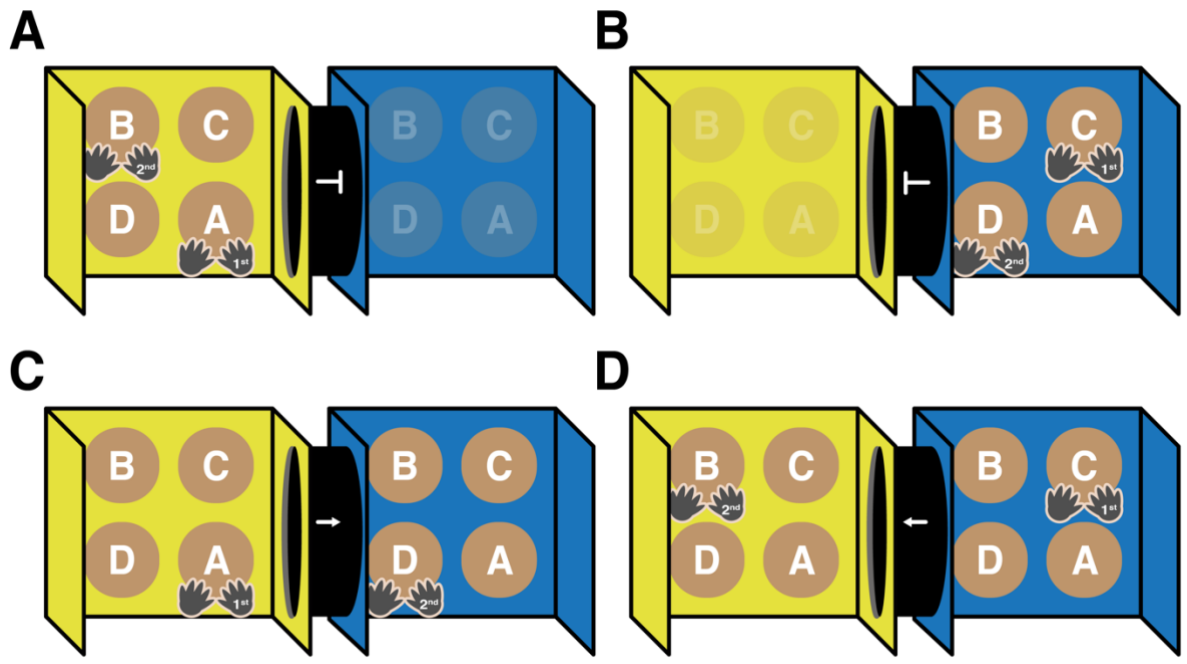


Figure 3.3. Schematic of the testing phases.

A) *and* ***B)*** *Describe the order of objects for the single context phase of the experiment.* ***A)*** *Shows the Yellow context sequence and* ***B)*** *Shows the Blue context sequence. 1st and 2nd hands indicate the correct order in which the objects needed to be touched. Arrows indicate the direction of movement. Faded boxes and objects indicate restricted access.* ***C)*** *and* ***D)*** *Describe the order of objects for the two context phase of the experiment.* ***C)*** *Shows the Yellow-Blue context sequence and* ***D)*** *Shows the Blue-Yellow context sequence.*

The animals were then tested on the two context sequences (Figure 3.3C-D), using the same approach as for single context sequences with no luring being included. The

threshold to pass was 30 out of 40 trials correct across two consecutive testing sessions.

If threshold performance was met, the next phase required the marmosets to perform the same two context sequences, under a more demanding trial structure, termed the alternating context phase. In this phase, the occurrence of Yellow-Blue and Blue-Yellow trial types was counterbalanced within sessions, rather than being consistent across an entire session. Each marmoset performed six trials per session, with trial types being intermixed to ensure that both types of sequences occurred within the same session. This counterbalancing specifically referred to the trial-by-trial alternation of context sequence direction, requiring animals to retrieve the appropriate sequence structure dynamically without relying on a fixed session-wide rule.

To familiarise the marmosets with the new trial structure, they underwent a brief retraining phase where correct sequences were reinforced as needed. Once stable performance was achieved, each marmoset was tested on alternating context trials, with a performance threshold set at 10 out of 12 trials correct over two consecutive testing sessions.

3.2.4.2. Cohort 2

For Cohort 2, training began similarly to Cohort 1 with the maze positioned on the bottom half of the home-unit. Marmosets were first trained on single context sequences (Figure 3.3A-B) where one context (either Yellow or Blue) was presented per session (10 trials per session). The training procedure mirrored that of Cohort 1; animals were first trained to approach the target objects, then to grasp them, with luring allowed throughout these stages. During the final stage of single context training, no luring was provided, matching the procedure for Cohort 1. One procedural change was that marmosets were capped at 20 trials per session, rather than having unlimited access for 10 minutes.

This led to the testing phase for single context sequences, where the number of trials per session was reduced to encourage sustained engagement and prevent boredom. To progress to the next phase, marmosets were required to achieve 17 out of 20 correct trials across two consecutive sessions.

Upon successful learning of single context sequences, marmosets progressed to the two context sequence phase. As in Cohort 1, animals were lured to the relevant objects only when necessary, and there were no defined correct or incorrect trials during training. The context providing access to the maze alternated between sessions. A procedural adjustment was that each session comprised 10 trials rather than 20, with progression criteria set at completing 17 out of 20 trials across two sessions. Following training, animals were tested on the two context sequences, again requiring 17 out 20 trials correct across two consecutive session to advance.

The alternating context phase for Cohort 2 was identical in structure to Cohort 1. Animals performed six trials per session, with trial types (Yellow-Blue and Blue-Yellow sequences) counterbalanced within sessions, rather than being fixed across sessions. After a brief retraining period where correct sequence were reinforced as needed, animals were tested, with performance thresholds again set at 10 out of 12 correct trials over two consecutive testing sessions.

A notable difference between cohorts was a modification to the maze's position during Cohort 2's experiment. Midway through training on the two context sequences, the maze was moved from the bottom to the top half of the home-unit in response to reduced voluntary engagement. Despite multiple re-habituation attempts (e.g., unsupervised maze access for an hour), interaction remained low until the relocation, after which engagement improved.

3.3. Results

3.3.1. Phase 1: Single context - temporal order sequence in one context

Initially, all marmosets learned a sequence that was relevant to a single context. There were two contexts and as such, two separate sequences to learn. For each sequence, this involved a pair of objects that needed to be interacted with in a specific order in a single context. For example, for the Yellow context, the correct order of choice was object A followed by object B (Figure 3.3A) and for the Blue context, the sequence to be learned was object C proceeded by object D (Figure 3.3B).

Learning of the two single context sequences was operationalised as performing a full sequence correctly in at least 30 out of 40 consecutive trials across two days for Cohort 1 (p = 0.001, binomial test, one-tailed), and in at least 17 out of 20 consecutive trials across two days for Cohort 2 (p = 0.001, binomial test, one-tailed). Out of the five animals that attempted to learn these sequences, all successfully met criterion (Figure 3.4A), with the average number of days undertaken by each marmoset to reach criterion being 5 days (mean: 4.6 days ± SD: 1.52, Figure 3.4B). Individual performance plots are illustrated in Figure 3.5.

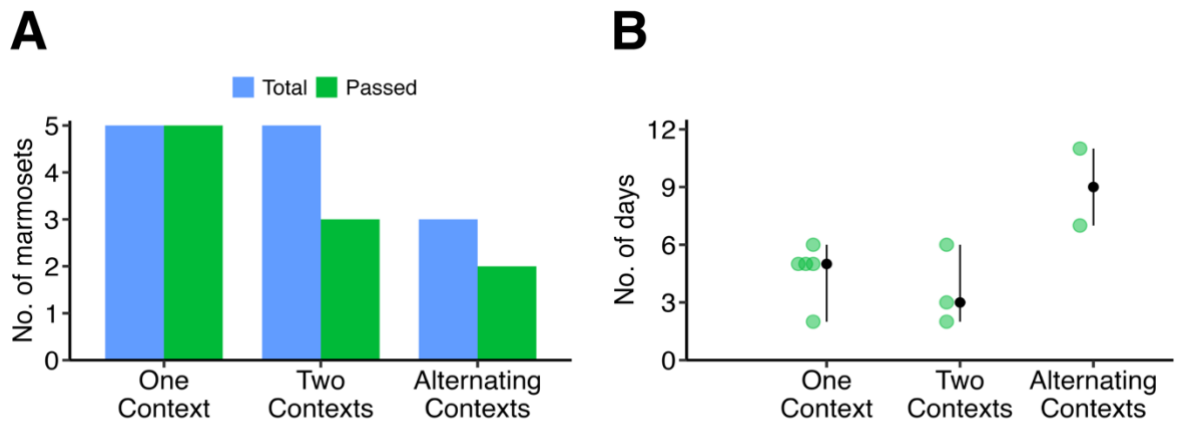


Figure 3.4. Number of marmosets and days needed to pass each testing phase.

A) *Shows the total number of marmosets for each phase (blue bars) and the number that successfully completed the phase (green bars).* ***B)*** *Displays the median number of days taken to complete each testing phase (black dots), with the number of days for each individual denoted by green dots*

3.3.2. Phase 2: Two context - temporal order sequence across two different contexts

As all five marmosets successfully learned the single context sequences, all five progressed to the next testing phase, where they were required to remember two sequences that now spanned across two different contexts. Figure 3.3C-D displays the two possible sequences: if transitioning from the Yellow context to the Blue context, the correct object order was object A in the Yellow context followed by object D in the Blue context (Figure 3.3C); if transitioning from the Blue context to the Yellow context, animals were required to interact with object C before choosing object B in the Yellow context (Figure 3.3D). Importantly, in this phase, each session presented only type of transition, with the direction (Yellow-Blue or Blue-Yellow) alternating across sessions rather than within them. For Cohort 1, the criterion was 30 or more trials correct out of 40 across two days; for Cohort 2, this was 17 out of 20 trials across two days.

Learning the sequences that spanned across the two contexts was achieved by the majority, with three out of five marmosets successfully reaching criterion across both cohorts (Figure 3.4A), taking, on average, 4 days to reach criterion (mean: 3.6 days ± SD: 2.08, Figure 3.4B). Individual performance plots are illustrated in Figure 3.5.

3.3.3. Phase 3: Alternating context - alternating sequence transitions in a session

Marmosets that successfully reached criterion for both single and two context learning, marmosets progressed to the final testing phase. During each session, six trials were randomly presented, exposing marmosets to both Yellow-Blue (Figure 3.3C) and Blue-Yellow (Figure 3.3D) sequences intermixed within the same session in a counterbalanced order (three trials of each type per session). Thus, unlike the two context phase, marmosets were now required to flexibly switch between different sequence transitions within a session. The performance criterion for success was 10 out of 12 trials correct across two consecutive days (p = 0.019, binomial test, one-tailed) for both cohorts. Two out of three marmosets successfully reached criterion when sequence type alternated within a given session (Figure 3.4A). However, the average number of days to reach criterion was higher than in previous phases (mean: 9.0 days ± SD: 2.83, Figure 3.4B), suggesting that switching contextual rules within a

session was achievable but presented a greater cognitive challenge. Individual performance plots are illustrated in Figure 3.5.

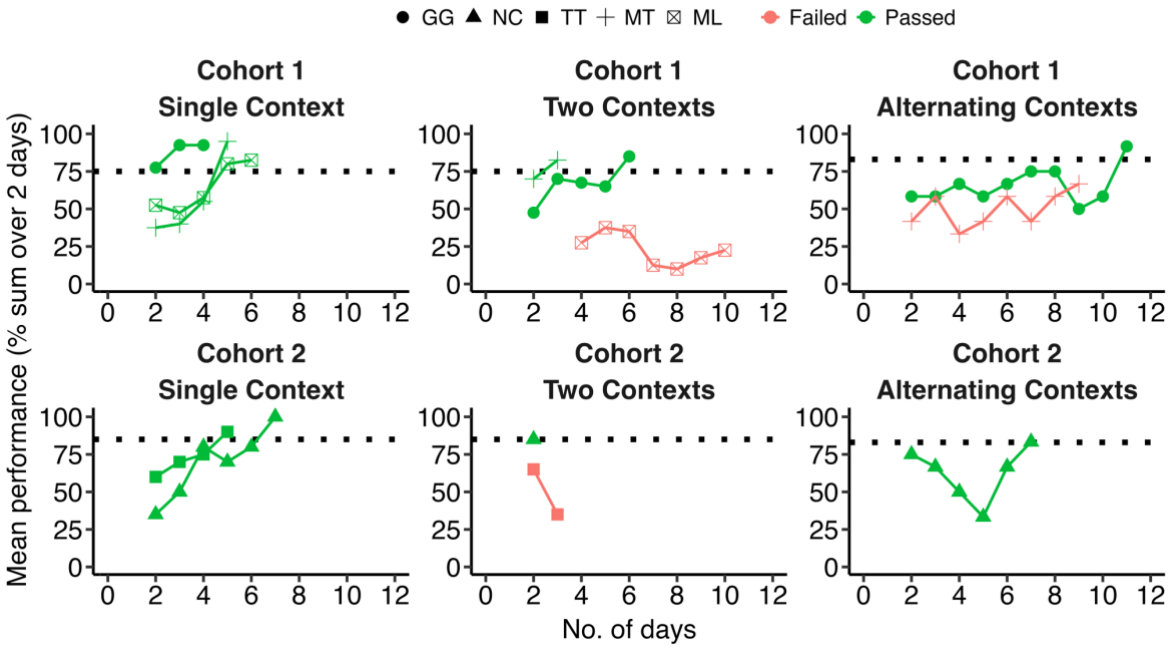


Figure 3.5. Individual performance for each marmoset for each testing phase.

Mean performance across testing days for individual marmosets from Cohort 1 (top row) and Cohort 2 (bottom row) in three testing conditions: Single Context (left column), Two Contexts (middle column), and Alternating Contexts (right column). Performance is plotted as the mean percentage of correct responses summed over 2-day blocks. Each line represents an individual marmoset, coded by animal ID (GG, NC, TT, MT, ML) and shape. Green lines indicate animals that passed criterion (black dotted line) relative to cohort and testing condition, and red lines indicate animals that failed to reach criterion. Some animals were removed from testing early (e.g., in the Two Context condition for Cohort 2). The x-axis denotes the number of days of testing, and the y-axis shows performance (% correct).

3.3.4. Comparison of error ratios for single context sequences

To further understand each marmoset's individual performance, error ratios were calculated for each subject at each of the three testing phases. Error ratios were defined as the number of errors made divided by the total number of trials attempted during a session per marmoset. Given that in a trial, two objects needed to be chosen and in a required order, two error types could occur: '1st Object' errors and '2nd Object'

errors. *1st Object* errors refer to trials when the first object chosen was incorrect and *2nd Object* errors refer to when the second object in the sequence was incorrect.

For the single context phase (Phase 1), a linear mixed effects model with error ratio as the dependent variable was applied. Error type (1st or 2nd Object errors) and individual marmoset were included as fixed effects, with a random intercept for testing date to account for session-level variability. An interaction term (error type × marmoset) was also included to assess whether the pattern of errors differed across animals.

The model revealed a significant main effect of error type (F (1, 29.6) = 6.38, p = 0.017), indicating that 1st and 2nd Object errors occurred at different rates. However, there was no significant main effect of marmoset (F (4, 29.3) = 0.754, p = 0.564) and no significant interaction between error type and marmoset (F (4, 29.6) = 1.21, p = 0.328), suggesting that this pattern was consistent across subjects (Figure 3.6A).

To further characterise the main effect, estimated marginal means were compared across the cohort. On average, 2nd Object errors occurred more frequently (mean: 0.216 ± SE: 0.037) than 1st Object errors (mean: 0.088 ± SE: 0.037, t (29.6) = 2.53, p = 0.017, d = 0.70). This pattern indicates that animals were more prone to making errors in the second step of the object sequence.

3.3.5. Comparison of error ratios for two context sequences

For the two context phase (Phase 2), a linear mixed-effects model with error ratio as the dependent variable was applied. Error type (1st or 2nd Object errors) and individual marmoset were included as fixed effects, with a random intercept for testing date to account for session-level variability. An interaction term (error type × marmoset) was also included to examine whether the error pattern differed across animals.

The model revealed a significant main effect of error type (F (1, 20.6) = 33.8, p < .001), indicating that 1st and 2nd Object errors occurred at different rates. There was also a significant main effect of marmoset (F (4, 19.5) = 4.26, p = 0.012) and a significant interaction between error type and marmoset (F (4, 20.6) = 4.23, p = 0.011), suggesting that the distribution of error types varied between animals (Figure 3.6B).

To further characterise the interaction, pairwise comparisons were conducted for each subject. Three marmosets showed significantly higher error ratios for 2nd Object errors

compared to 1st Object errors (GG: t (20.6) = 2.75, p = 0.012, d = 1.59), MT: t (20.6) = 4.72, p < .001, d = 3.85), and TT: t (20.6) = 7.07, p < .001, d = 3.16, Figure 3.6B). For ML and NC, however, the differences between error types were not significant (Figure 3.6B).

3.3.6. Comparison of error ratios for alternating context sequences

For the alternating context sequence phase (Phase 3), a linear mixed-effects model with error ratio as the dependent variable was applied. Error type (1st or 2nd Object errors) and individual marmoset were included as fixed effects, with a random intercept for testing date to account for session-level variability. An interaction term (error type × marmoset) was also included to examine whether the error pattern differed across animals.

The model revealed a significant main effect of error type (F (1, 18) = 25.4, p < .001), indicating that 1st and 2nd Object errors occurred at different rates. However, there was no significant main effect of marmoset (F (2, 18) = 0.578, p = 0.571), and no significant interaction between error type and marmoset (F (2, 18) = 1.79, p = 0.195, Figure 3.6C).

To further examine the main effect, estimated marginal means were compared across the cohort. On average, 2nd Object errors occurred more frequently (mean: 0.363 ± SE: 0.043) than 1st Object errors (mean: 0.055 ± SE: 0.043, t (18) = 5.04, p < .001, d = 1.60). This pattern indicates that animals were more prone to making errors in the second step of the object sequence (Figure 3.6C).

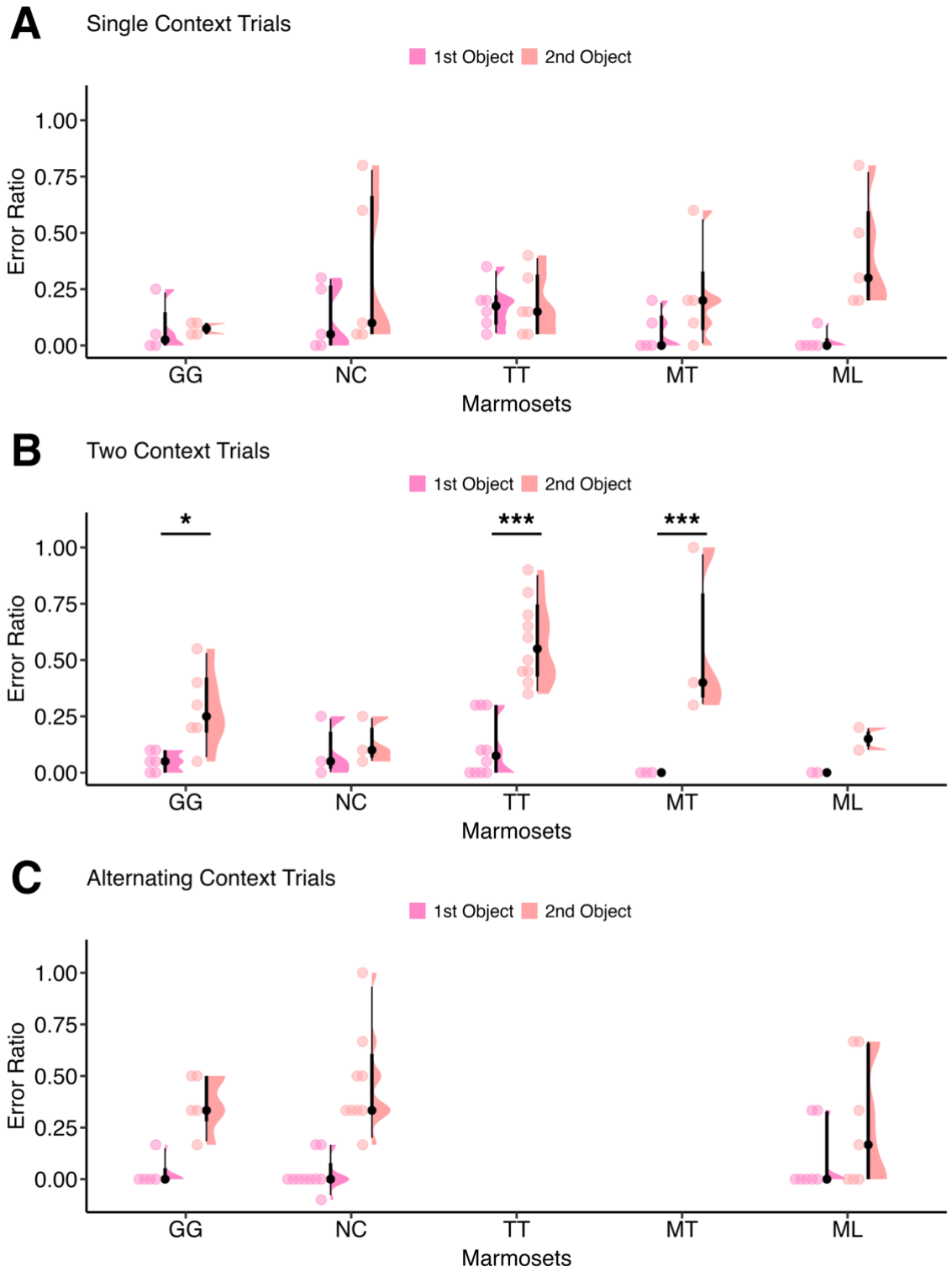


Figure 3.6. Comparison of error rates for the three phases of the experiment.

A) *Shows error rates for the individual marmosets split by error type, 1st Object errors and 2nd Object errors for the single context phase.* ***B)*** *Shows the same error rates for the two context phase and* ***C)*** *Shows the same error rates for the alternating context*

*phase. Black dots represent the median error ratio, with black lines indicating the range. The shaded regions display the data distribution. Coloured dots relate to individual error ratios in a given session. Significance is indicated by *** ($p < .001$) or * ($p < .05$).*

3.3.7. Comparison of error ratios related to motivation and cognition

During both training and testing phases of the experiment, some animals exhibited an unwillingness to complete sequences or choose an object upon entering the maze. Instead, these animals favoured to leave the maze, effectively aborting the trial. For the overall analysis above, these instances were categorised as incorrect trials, grouping them with other errors related to mistakes during object selection. To more clearly differentiate these trial types, errors resulting from mistakes in object selection were reclassified as 'Cognition' errors, while those due to unwillingness to participate were categorised as 'Motivation' errors.

The frequency of Cognition and Motivation errors across each testing phase was then analysed using a linear mixed-effects model, with error ratio as the dependent variable. Error type (Cognition or Motivation) and phase (Single context, Two context, and Alternating context) were included as fixed effects, with a random intercept for individual marmosets to account for inter-subject variability. An interaction term (phase × error type) was also included to examine whether the pattern of errors differed across phases and error types.

The analysis revealed a significant main effect of error type (F (1, 282.1) = 30.2, p < .001), with significantly more Motivation errors (mean: 0.16 ± SE: 0.17) than Cognition errors (mean: 0.04 ± SE: 0.17). However, no significant effect of phase was observed (F (2, 182.3) = 1.43, p = 0.243), nor was there a significant interaction between phase and error type (F (2, 282.1) = 2.04, p = 0.132, Figure 3.7A). Estimated marginal means comparison further confirmed the significant difference between Motivation and Cognition errors (t (282) = 5.50, p < .001, d = 0.645, Figure 3.7A).

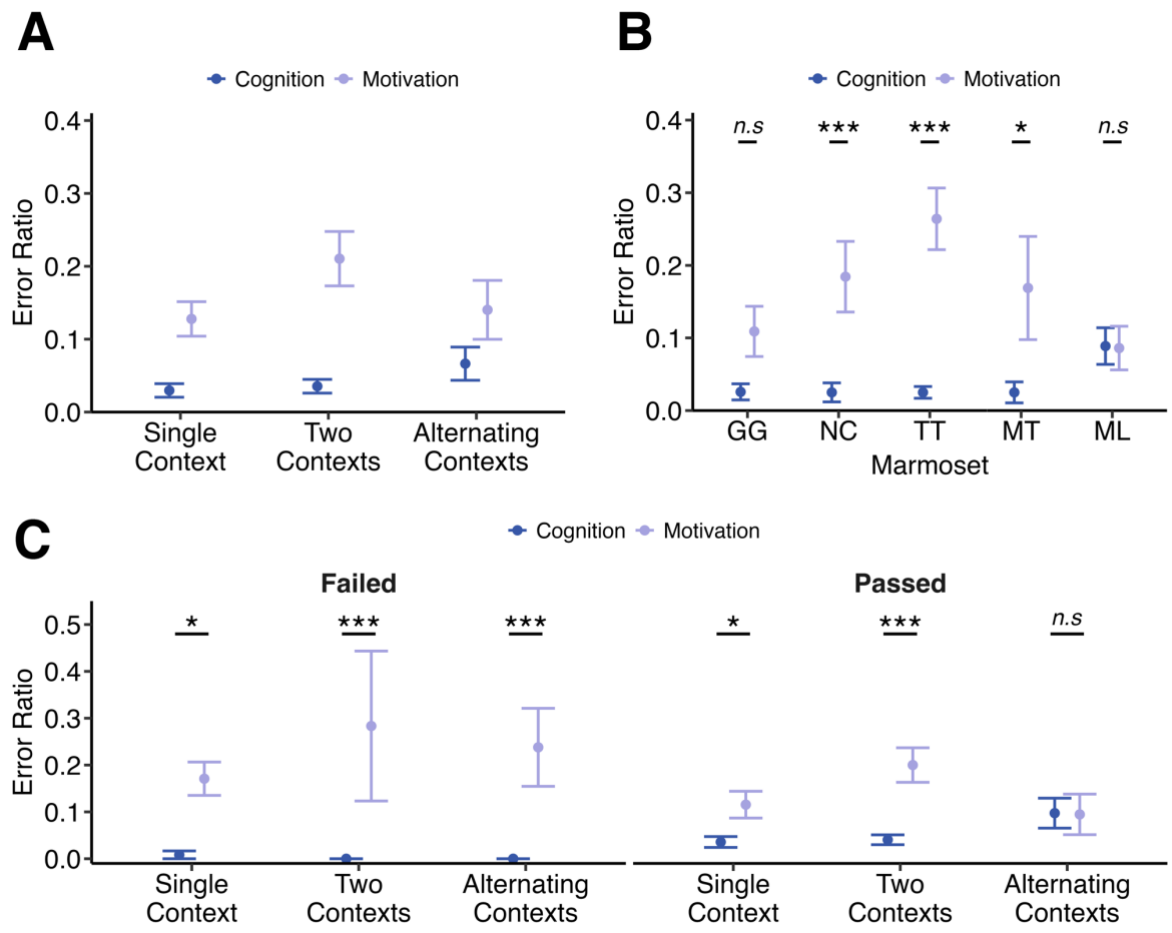


Figure 3.7. Comparison of motivation and cognitive errors, split by phase and marmoset.

A) *Shows error rates for Cognition and Motivation errors split by each of the three phases of the experiment.* ***B)*** *Shows the same error rates across the entire experiment, split by individual marmoset.* ***C)*** *Shows error rates for each phase, split into marmosets that passed the phase and those that failed to reach criterion. Dots represent the mean and error bars display ± SE. Significance is indicated by *** ($p < .001$), * ($p < .05$) or n.s ($p > .05$).*

To assess whether this pattern persisted at the individual level, the error types for each marmoset across all phases of the experiment were analysed. A linear mixed-effects model was conducted with individual marmoset (5 animals) and error type (Cognition and Motivation) as fixed effects, and phase included as a random intercept to account for inter-subject variability. An interaction term was also included (marmoset × error type) was also included to examine whether the pattern of errors differed across animals and error types.

The model did not show a significant main effect of marmoset (F (4, 225) = 1.48, p = 0.210), but did show a significant main effect of error type (F (1, 280) = 33.5, p < .001) and a clear interaction between error type and marmoset (F (4, 280) = 4.66, p = 0.001).

To further examine the effect of the interaction, post-hoc comparisons were conducted for each marmoset, comparing the error rates of Cognition versus Motivation errors. Marmoset TT, which failed to pass the Two context phase (Phase 2), exhibited significantly more Motivation errors (mean: 0.27 ± SE: 0.03) than Cognition errors (mean: 0.03 ± SE: 0.03 t (280) = 5.45, p < .001, d = 1.36, Figure 3.7B). Similarly, marmoset NC, which advanced to the final phase (Alternating contexts), also showed significantly more Motivation errors (mean: 0.18 ± SE: 0.03) than Cognition errors (mean: 0.03 ± SE: 0.03, t (280) = 3.51, p < .001, d = 0.908, Figure 3.7B). As well, marmoset MT showed a significant increase in Motivation error ratios (mean: 0.17 ± SE: 0.05) compared to Cognition error ratios (mean: 0.03 ± SE: 0.05, t (280) = 2.32, p = 0.021, d = 0.819, Figure 3.7B). However, this result might not fully reflect MT's performance, as the animal was removed early from experimentation due to their refusal to enter the maze after only a few sessions, potentially preventing further motivational errors from emerging. It should be noted, however, that the two marmosets that successfully learned all sequences and passed all phases of the experiment (GG and ML) showed no statistical difference between Motivation and Cognition error ratios (GG: t (280) = 1.78, p = 0.077, d = 0.474, and ML: t (280) = 0.070, p = 0.944, d = 0.016, Figure 3.7B).

To further assess the impact of whether success in reaching criterion for each phase affected the proportion of each error type, a linear mixed-effects model was conducted that factored in whether the animal passed or failed to reach criterion for each phase. As such, the model included error type (Cognition and Motivation), phase (all three) and success rate (passed or failed) as fixed effects, and included marmoset as a random intercept to account for inter-subject variability. An interaction term was also included (error type × phase × success rate) was also included to examine whether the pattern of errors differed across animals, error types and success rates.

The model showed no main effect of phase (F (2, 72.9) = 1.61, p = 0.210) or success rate (F (1, 94.5) = 0.023, p = 0.881), but did identify a main effect of error type (F (1, 276) = 33.6, p < .001, Figure 3.7C). In addition, there was a significant interaction

between error type and success rate (F (1, 276) = 7.94, p = 0.005), but not for the other interactions tested (phase × error type: F (2, 276) = 1.37, p = 0.256; phase × success rate: F (2, 22.9) = 0.316, p = 0.732), including the three-way interaction (F (2, 276) = 0.989, p = 0.374).

To further explore the significant interaction between error type and success rate, pairwise contrasts were conducted between Cognition and Motivation-related error rates within each phase and success rate group (Passed versus Failed). These comparisons revealed a consistent pattern of higher error rates for Cognition errors relative to Motivation errors among animals that failed to reach criterion.

Notably, this pattern likely reflects the underlying motivational state of the animals: individuals with elevated Motivation errors may have exited trials before making an object selection, thereby reducing their opportunity to accrue Cognition errors. In contrast, animals who remained engaged but nevertheless struggled with task demands exhibited a relatively higher proportion of cognition-related mistakes.

In the Single context phase, failed animals showed significantly more Cognition errors than Motivation errors (t (276) = - 2.25, p = 0.025, d = - 0.92). This pattern was also evident among animals that passed, though the difference was attenuated (t (276) = - 2.07, p = 0.040, d = - 0.45, Figure 3.7C).

In the Two contexts phase, failed animals again exhibited significantly more Cognition errors than Motivation errors (t (276) = - 2.77, p = 0.006, d = - 1.60), and this difference remained robust in the group that passed (t (276) = - 4.13, p < .001, d = - 0.90, Figure 3.7C).

In contrast, in the Alternating contexts phase, only failed animals demonstrated a significant bias toward Cognition errors (t (276) = - 3.56, p < .001, d = - 1.34). For animals that passed, no significant difference in error type was observed (t (276) = 0.058, p = 0.954, d = 0.02, Figure 3.7C).

These results confirm that the relative proportion of error types was modulated both by success rate and task phase, with cognition-related errors disproportionately elevated in lower-performing animals, particularly under conditions involving context change.

3.3.8. Comparison of error ratios related to context and failure rate

To investigate why marmosets struggled with the two context phase, error rates were assessed for all marmosets in relation to both context and success rate. Specifically, a linear mixed effects model was used to examine how context, success rate and error type influenced error ratio. This model included random intercepts for each marmoset to account for individual variability in baseline error rates.

Here, context refers to whether the trial began in the Yellow context and then transitioned to the Blue context (referred to as 'Yellow-to-Blue' trials) or started in the Blue context and transitioned to the Yellow context (termed 'Blue-to-Yellow' trials). Failure rate was a two-level factor based on whether or not each marmoset reached criterion ('Passed') or did not ('Failed'). As well, error type was further divided into four categories, '1st Incomplete' refers to trials where marmosets entered the maze but did not make a choice, '2nd Incomplete' refers to trials in which the first object selected was correct but the second object in the sequence was not selected at all. '1st Object' and '2nd Object' errors are the same as defined previously.

The results of the model revealed no significant main effect of context (F (1, 79.1) = 3.06, p = 0.084), and no significant main effect of success rate on error ratio (F (1, 1.68) = 11.63, p = 0.097). A significant main effect of error type was seen (F (3, 77.1) = 57.0, p < .001) along with a significant interaction effect between context and error type (F (3, 77.1) = 6.17, p < .001), context and success rate (F (1, 79.1) = 4.42, p = .039), and error type and success rate (F (3, 77.1) = 24.4, p < .001). Interestingly, there was no significant main effect of the three-way interaction (F (3, 77.1) = 0.638, p = 0.594).

Pairwise comparisons were conducted to assess the difference in error ratio for the four error types for both context and failure rate.

In the Yellow-Blue context, for animals that failed to progress, 2nd Incomplete errors were performed significantly more often than all other error types (Figure 3.8A). This included 1st Incomplete errors (t (77.1) = 11.15, p < .001, d = 5.96), 1st Object errors (t (77.1) = 12.49, p < .001, d = 6.68), and 2nd Object errors (t (77.1) = 11.95, p < .001, d = 6.39). No significant differences were observed among the remaining comparisons.

In the Blue-Yellow context, for animals that failed to progress, there was a significant difference in the distribution of error types (Figure 3.8B). Specifically, 2nd Incomplete errors were made significantly more often than 1st Incomplete errors (t (77.1) = 6.53, p < .001, d = 3.77), 1st Object errors (t (77.1) = 6.67, p < .001, d = 3.85), and 2nd Object errors (t (77.1) = 6.96, p < .001, d = 4.02). No significant differences were observed among the other error types.

In the Yellow-Blue context (Figure 3.8C), for animals that successfully passed the two context phase, 2nd Incomplete errors were performed significantly more often than 1st Incomplete errors (t (77.1) = - 2.90, p = 0.025, d = - 1.68), 1st Object errors (t (77.1) = 3.34, p = 0.007, d = 1.93), and 2nd Object errors (t (77.1) = 3.19, p = 0.011, d = 1.84). No significant differences were observed among the remaining error types.

In the Blue-Yellow context (Figure 3.8D), for animals that progressed, no significant differences in error type were observed. Specifically, 2nd Incomplete errors were not significantly more frequent than 1st Incomplete (t (77.1) = 1.27, p = 0.584, d = 0.80), 1st Object (t (77.1) = 0.636, p = 0.920, d = 0.40), or 2nd Object errors (t (77.1) = - 0.160, p = 0.999, d = - 0.10). All other pairwise comparisons were similarly not significant: 1st Incomplete vs. 1st Object (t (77.1) = - 0.636, p = 0.920, d = - 0.40), 1st Incomplete vs. 2nd Object (t (77.1) = - 1.43, p = 0.485, d = - 0.90), and 1st Object vs. 2nd. Object (t (77.1) = - 0.795, p = 0.857, d = - 0.50).

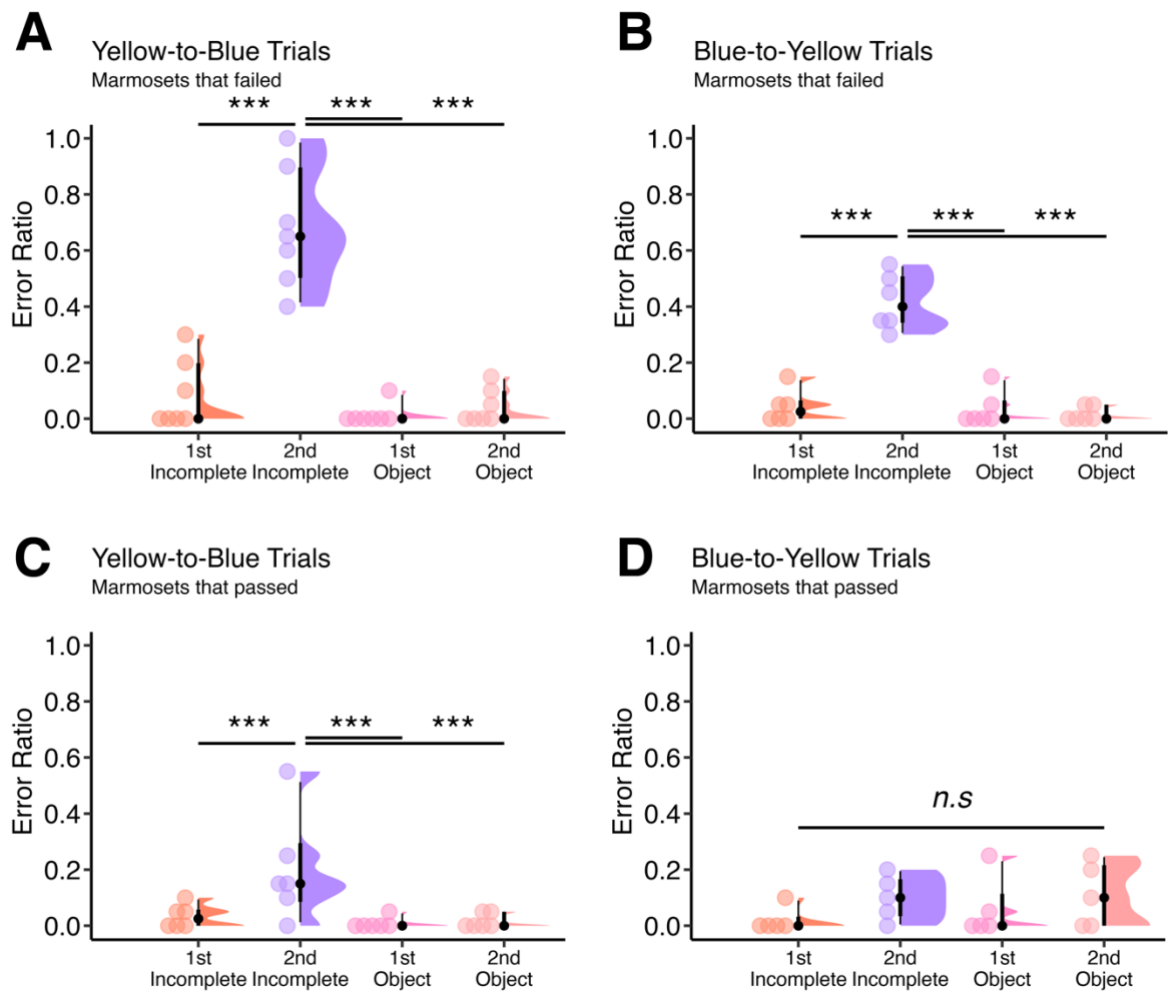


Figure 3.8. Comparison of error types for two context trials, split by context and failure rate.

A) *and* ***B)*** *Show error rates for marmosets that failed the phase, with* ***A)*** *Focusing on Yellow-Blue trials and* ***B)*** *Showing Blue-Yellow trials.* ***C)*** *and* ***D)*** *Show error rates for marmosets that passed, with* ***C)*** *Displaying the results for Yellow-Blue trials and* ***D)*** *showing the rates for Blue-Yellow trials. Black dots represent the median error ratio, with black lines indicating the range. The shaded regions display the data distribution. Coloured dots relate to individual error ratios in a given session. Significance is indicated by *** (p < .001), * (p < .05) or n.s (p > .05).*

3.3.9. Comparison of cognitive error ratios for two context sequences

To further understand the prevalence of cognitive errors on performance during the two context phase of the experiment, errors were further divided and subcategorised. 1st Object and 2nd Object errors (Cognition errors) were separated from Motivation errors (1st Incomplete and 2nd Incomplete errors) and categorised into three error types

displayed in Table 3.1. Error types that could be made included, 'Order' errors, when the animal chose an object that was relevant to the current context, but it was not the correct object in the sequence, 'Context' errors, when the animal chose an object that followed the correct sequence order but belonged to the opposite context, and finally, when the animal chose an object that was incorrect in both sequence order and context, this was regarded as a 'Full' error.

Table 3.1 Error types for cognitive errors in two and alternating context trials.

Abbreviations: + denotes a correct response

Context	**Object**	**Choice Position**	
		1st	2nd
Blue-to-Yellow (B-Y)	A	Context	Order
	B	Full	+
	C	+	Full
	D	Order	Context
Yellow-to-Blue (Y-B)	A	+	Full
	B	Order	Context
	C	Context	Order
	D	Full	+

A linear mixed-effects model was conducted with error type (Order, Context, Full), context direction (Blue-to-Yellow, Yellow-to-Blue), and failure rate (Passed, Failed) as fixed effects, including their three-way interaction. Marmoset identity was included as a random intercept to account for inter-individual variability.

The linear mixed-effects model revealed a significant main effect of error type (F (2, 60.04) = 4.75, p = 0.012, Figure 3.9A), indicating that the type of error made (Order, Context, or Full) significantly influenced performance. Specifically, Context errors (mean: 0.32 ± SE: 0.09) were more prevalent than Full errors (mean: 0.02 ± SE: 0.09, t (60) = 2.98, p = 0.012, d = 0.843), however, not significantly different to Order errors

(mean: 0.10 ± SE: 0.09, t (60) = 2.19, p = 0.082, d = 0.620). In addition, the prevalence of Order errors was not significantly different to Full errors (t (60) = 0.787, p = 0.712, d = 0.223). These findings suggest that the marmosets were more likely to make context-based errors rather than Full errors, indicating that while they could remember the correct sequence of objects, they were less successful at incorporating the context into their decision-making. This implies that the animals may have relied more on the order of objects than on the context in guiding their choices during the task.

In contrast, there were no significant main effect of context (F (1, 60.46) = 0.072, p = 0.790, Figure 3.9B) or failure rate (F (1, 2.55) = 0.014, p = 0.915) Figure 3.9C), suggesting that neither the direction of context change nor whether animals passed or failed the phase significantly influenced overall error frequency when considered in isolation. None of the two-way interactions reached significance. However, the three-way interaction between error type, context, and failure rate approached significance (F (2, 60.0) = 2.70, p = 0.075). Further inspection of the model coefficients revealed a significant simple effect: animals in the Yellow-to-Blue context who passed exhibited more Order errors (β = 0.320, 95 % CI [- 0.014, 0.653], t (60) = 2.31, p = 0.024, Figure 3.9D). This suggests that, although overall performance was successful, marmosets in this particular context continued to struggle with determining the correct object order.

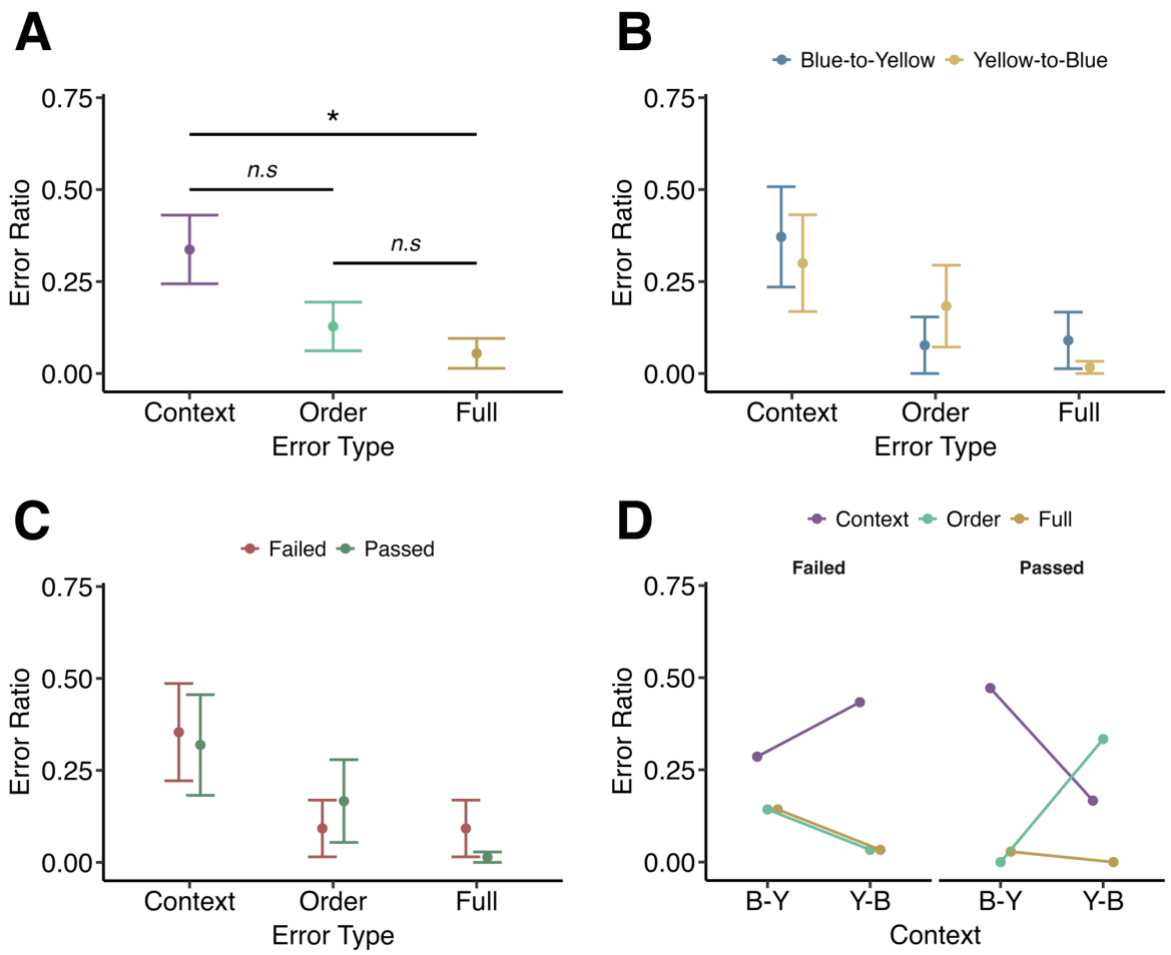


Figure 3.9. Comparison of cognition errors for two context trials, split by context and failure rate.

A) *Error ratios for three error types: (1) Context errors, where the chosen object adhered to the correct sequence order but belonged to the opposite context; (2) Order errors, where the animal selected an object relevant to the current context but incorrect in the sequence; and (3) Full errors, where the selected object was incorrect in both sequence order and context.* ***B)*** *Error ratios across two context conditions (Yellow-Blue and Blue-Yellow) for the three error types.* ***C)*** *Error ratios for the three error types grouped by marmosets that either Failed or Passed.* ***D)*** *Error ratios broken down by both context and failure rate. Dots represent the mean and error bars display ± SE. Significance is indicated by * (p < .05) or n.s (p > .05).*

3.3.10. Comparison of cognitive error ratios for alternating context sequences

For the alternating context phase, a linear mixed-effects model was conducted with error type (Order, Context, Full), context direction (Blue-to-Yellow, Yellow-to-Blue), and failure rate (Passed, Failed) as fixed effects, including their three-way interaction.

Marmoset identity was included as a random intercept to account for inter-individual variability.

The model showed no significant main effect of error type (F (2, 145.6) = 0.841, p = 0.433, Figure 3.10A), or context (F (1, 145.6) = 0.014, p = 0.905, Figure 3.10B). The main effect of failure rate was also not significant (F (1, 41.1) = 0.834, p = 0.366, Figure 3.10C). Furthermore, none of the two-way interactions reached statistical significance: the interaction between error type and context (F (2, 145.6) = 0.866, p = 0.423); between error type and failure rate (F (2, 145.6) = 1.29, p = 0.278; and between context and failure rate (F (1, 145.6) = 0.917, p = 0.340). In addition, the three-way interaction between error type, context, and failure rate proved not to be significant (F (2, 145.6) = 2.22, p = 0.112, Figure 3.10D).

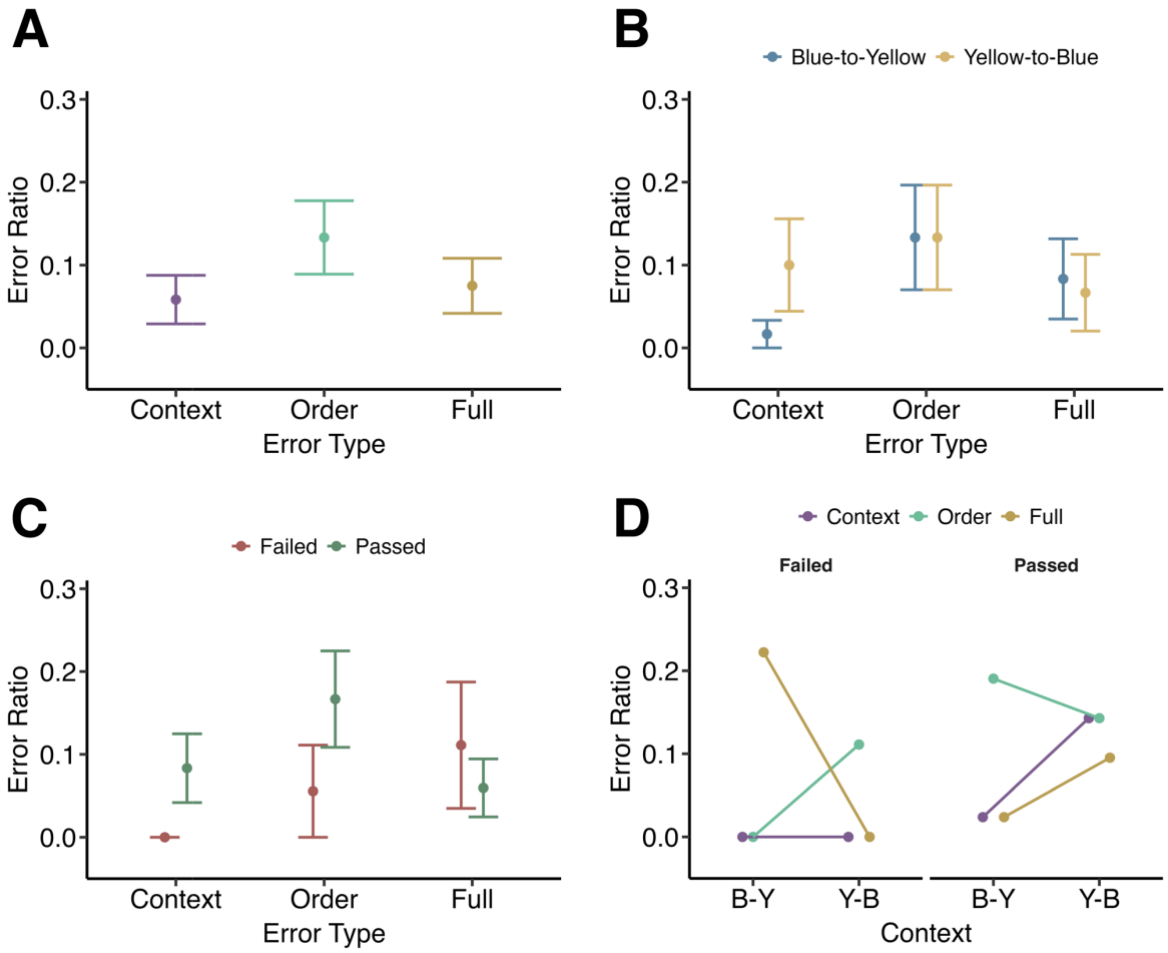


Figure 3.10. Comparison of cognition errors for alternating context trials, split by context and failure rate.

A) *Error ratios for three error types: (1) Context errors, where the chosen object adhered to the correct sequence order but belonged to the opposite context; (2) Order*

errors, where the animal selected an object relevant to the current context but incorrect in the sequence; and (3) Full errors, where the selected object was incorrect in both sequence order and context. ***B)*** *Error ratios across two context conditions (Yellow-Blue and Blue-Yellow) for the three error types.* ***C)*** *Error ratios for the three error types grouped by marmosets that either Failed or Passed.* ***D)*** *Error ratios broken down by both context and failure rate. Dots represent the mean and error bars display ± SE. Significance is indicated by n.s (p > .05).*

3.4. Discussion

This study examined how five marmosets learned and performed temporal context-dependent sequences both within a single context and across two contexts. The findings from the experiment shed light on the cognitive, motivational and ecological factors that influence a marmoset's ability to learn and adapt overlapping context-dependent sequences. Ultimately, these results are important for understanding the complexities of marmoset cognition, particularly in tasks that require integration of multiple modalities such as object identity and environmental context.

Initially, the five marmosets were trained to interact with pairs of objects in a specific temporal order within a single context. All five marmosets successfully reached the criterion, demonstrating their ability to efficiently encode and retrieve temporal sequences efficiently when these sequences were presented in a stable and familiar context. This finding aligns with research in humans, which shows that a familiar spatial context enhances memory recall (Godden & Baddeley, 1975; Robin & Moscovitch, 2014). For example, when human participants were tested in the same place where they originally learned a piece of information, their ability to recall that same information significantly improved.

Next, the marmosets were tested on sequences that spanned across two contexts. This required them to adjust to a new context mid-sequence and alter their choice of object accordingly. Overall, performance declined with only three out of five marmosets successfully reaching the criterion. These results suggest that the introduction of a mid-sequence contextual shift proved challenging for the marmosets and impaired their ability to recall the correct order of objects accurately. Further analysis revealed that this difficulty stemmed more from a lack of motivation than from a cognitive inability to recall the sequences. Specifically, the marmosets often chose not to transition between contexts, instead favouring to leave the apparatus mid-trial, which caused the trials to be aborted and be classified as a failure. These observations indicate that the primary challenge lay in maintaining task engagement rather than in memory recall itself.

Interestingly, error rates revealed that motivation was influenced by the direction of context transitions. Specifically, when the animals were required to move from the Yellow context to the Blue context, the number of aborted trials was consistently high,

regardless of whether the marmoset eventually passed the phase. Conversely, when transitioning in the opposite direction – from Blue to Yellow context – motivation levels varied significantly. Marmosets that failed to progress demonstrated much lower motivation compared to those that passed. Notably, the marmosets that succeeded exhibited no difference in cognitive or motivational errors when transitioning from Yellow to Blue context.

This finding is particularly intriguing, as it suggests that the Yellow context contained a motivating factor absent in the Blue context. Considering the setup of the apparatus, it is understandable that motivation to enter the second context was low in general. The second context lacked a clear exit point, which likely amplified risk aversion, as the marmosets avoided entering the second context for the majority of the trials. And yet, the marmosets' willingness to occasionally enter the Yellow context despite these concerns suggests the presence of a factor that occasionally overrode their risk aversion.

One such factor might be differences in light levels. Due to the opacity of the paint, the Blue context was much darker than the Yellow context. This reduced visibility may have made the marmosets feel trapped, as they had more difficulty seeing outside of the apparatus. Marmosets, being highly social animals, exhibit social facilitation when foraging. In groups, they are more effective at locating and exploiting food sources due to increased vigilance against predators (Fuente et al., 2022; Rogers et al., 2018). In the Yellow context, even in the absence of their conspecific partner, the marmosets may have felt less threatened and more willing to take risks for additional rewards.

Moreover, it is possible they could see their partner through the semi-transparent walls of the Yellow context, providing reassurance and reducing perceived risk. This is particularly likely, as in the wild the main predators of the *Callitrichidae* family are aerial raptors (Ferrari & Ferrari, 1990), making it disadvantageous to remain exposed without adequate cover unless reassured by the presence of a group member. Indeed, marmosets have developed a sophisticated system of alarm calls specifically triggered by the presence of aerial raptors. These calls serve to alert conspecifics to the danger and prompt them to seek refuge in denser vegetation (Bezerra et al., 2008).

Another potential factor impacting motivation relates to the ecological validity of the maze's placement within the testing environment. The maze was initially positioned at

the bottom of the home-unit so that dividers could be inserted, keeping one animal in the ‘testing area’ below while still allowing the remaining members of the group to occupy the space above. In the wild, however, marmosets live in dense foliage and are primarily arboreal, rarely descending to the ground (Schiel & Souto, 2017). This setup may have made the participating animal feel both threatened and vulnerable, being alone and so low to the floor, which likely reduced their motivation considerably. This issue was addressed mid-way through testing Cohort 2 by moving the maze to the top half of the home-unit. While this adjustment had mixed effects, with some individuals, like marmoset MT, continuing to refuse participation, it raises the possibility that the lack of motivation stemmed not just from the maze’s placement but from the apparatus itself.

This consideration of motivational factors is particularly important in comparative cognition research, as it challenges the assumption that marmosets are inherently more difficult to train than other nonhuman primates, such as macaques (Marx, 2016). This misconception likely arises from the use of experimental paradigms designed for other species, which are not ecologically suited to marmosets. For instance, traditional maze paradigms, typically designed for rodents, do not align with the natural behaviours and environmental interactions of marmosets, who are arboreal and rely on different environmental cues for navigation and foraging (Prins et al., 2017; Ngo et al., 2022; Hilário & Ferrari, 2015).

In terms of cognitive errors, an in depth analysis highlighted that marmosets struggled with context, often selecting the correct object for the temporal position but one that was relevant to the opposite context. This pattern was observed in both successful and unsuccessful animals, and across both context types, suggesting that, regardless of their level of understanding, marmosets faced an inherent difficulty in anchoring correct object choices to the appropriate contextual cues. These findings indicate that contextual memory, posed a significant hurdle for the marmosets.

The types of error produced suggest that the animals tended to complete sequences as though the context had not changed or was irrelevant. For example, in a trial involving a Blue-to-Yellow context transition, the marmosets were more likely to continue the same sequence (e.g., from object C to D) across the two contexts, despite the shift. This behaviour indicates a lack of awareness of the contextual change mid-

trial. One might instead expect the animals to perceive the transition as a new trial and ‘reset’ the sequence to align with the new context (e.g., transitioning from object C to A in a Blue-to-Yellow context shift). Collectively, these findings suggest that the contextual shift was not sufficiently salient to the marmosets.

In the final phase of the experiment, where marmosets were required to alternate between two context-dependent sequences within the same session, similar difficulties emerged, this time accompanied by an increase in the number of order errors. The increase in order errors compared to context errors, suggests that continuous and unpredictable shifting may have overwhelmed the animals' ability to maintain accurate sequences. Only two of the three tested marmosets were able to recall the correct sequences and reach threshold performance. Furthermore, the time taken to reach criterion was considerably longer compared to the previous two phases, further emphasising the heightened difficultly the marmosets experienced. This is surprising as marmosets’ abilities in reversal learning exceed expectations based on brain size and surpass the ability of other New World monkeys, including tool-using capuchins (Strasser & Burkart, 2012). It is worth mentioning that marmosets can also be trained to use tools with extensive training (Yamazaki et al., 2011).

The importance of order is already a fundamental part of a marmoset’s natural foraging behaviour. Although originally documented in another member of the *Callitrichidae* family, pygmy marmosets (*Callithrix pygmaea*) demonstrate temporal understanding and awareness of event sequences through their food-extraction behaviour. Tree exudates are the primary component of a marmoset’s diet with approximately 30 % of their daily activity spent gouging for tree gum (Power & Oftedal, 1996). For the gum to be available for consumption, however, marmosets must follow a multi-step process. First, they locate a suitable tree and gouge the trunk with their specially adapted incisors, prompting the exudate to flow – albeit at a rate too slow for immediate consumption. As such, marmosets typically gouge multiple holes in several trees before returning to their nesting areas. To collect the exudate, the marmosets are required to remember the location of the trees they gouged and how long ago they gouged them. Such behaviours indicate not only the applied use of spatial memory but also an intrinsic understanding of time-dependent sequences.

In a simulated foraging task, marmosets were tested on a win-shift strategy, whereby they had to remember which sites they had already visited and avoid revisiting those sites in subsequent trials (MacDonald et al., 1994). The marmosets tested performed at above-chance levels, despite making many errors in re-visiting the sites that had previously contained food. Consequently, the authors proposed that marmosets may possess a generalised spatial memory ability yet have personal biases for particular strategies. This may explain the preference for one context transition shown in the alternating context phase of the present study. Results from the current study showed that even successful marmosets made fewer errors all round when the context transitioned from Blue to Yellow than vice versa.

A subsequent study by Izumi et al. (2013), which demonstrated that marmosets were capable of spontaneous alternation in a Y-maze through use of spatial cues, proposed that the increase of errors in the win-shift foraging experiment (MacDonald et al., 1994) described above, was due to the need of inhibitory control. In other words, it might have been challenging for the marmosets to suppress their natural tendency to revisit previously baited sites. Similarly, in the current study, this difficulty may extend to the previously baited objects, potentially accounting for the higher error rates observed during multiple context transitions. These transitions likely required greater inhibitory control to resolve interference between competing sequence memories.

To better understand the biases seen in the study, further research could investigate whether they arise from factors such as contextual salience, associative learning differences, or variations in motivation. One promising avenue for future work might involve designing tasks more closely aligned with a marmoset's natural behaviour. For example, given the marmosets' preference for remaining high off the ground and their reliance on clinging to tree trunks, a vertical maze could better suit their instincts. A spatial navigation task requiring upward climbing toward a reward, also mimicking their gouging behaviour, might provide a more ecologically valid framework for studying marmoset memory processes.

Such an approach would also offer a distinct advantage over traditional primate tasks involving head-fixation or touchscreens: the freedom of movement. This mobility could enhance hippocampal function by delivering richer sensory input regarding the contextual environment (Vann et al., 2003). These sensory inputs, often absent in

restrained or head-fixed conditions, may provide critical contributions to spatial memory and sequence learning (Erickson et al., 2011; Zemla & Basu, 2017), making a vertical task an invaluable tool for future studies.

In summary, this study highlights the complex interplay between cognitive, motivational and ecological factors in marmoset behaviour during learning of a task that requires the integration of both context and sequence memory. While the marmosets demonstrated proficiency in learning object sequences within a single context, their performance declined when sequences spanned across multiple contexts. Motivational challenges, particularly task engagement, were a significant factor in these difficulties, alongside the inherent cognitive demands of switching between different contexts. The results suggest that context switching presents a greater challenge than sequence learning itself, potentially due to the fast rate in which animals were expected to switch between the two different contextual conditions. These findings reinforce the importance of designing tasks that align with the ecological behaviours of marmosets, and the problems that arise if not done so correctly. Ultimately, the study provides valuable insight into marmoset cognition and underscores the need to consider motivational and ecological validity when assessing cognitive performance in comparative research.

Chapter 4: Behavioural Assessment of Context-Guided Sequence Memory in Rhesus Macaques

4.1. Introduction

Episodic memory involves the capacity to recall events across multiple environments, integrating the details of 'what' occurred, 'where' it happened, and 'when' it took place (Tulving, 1983). While humans excel in this process, there is ongoing debate about whether nonhuman animals possess a comparable ability (see Section 1.2). Researchers instead propose that nonhuman animals exhibit 'episodic-like' memory, a concept distinct from human episodic memory in that it does not necessarily require the subjective experience of recollection. Instead, episodic-like memory allows animals to recall specific events without conscious awareness of those memories (Eacott & Norman, 2004). Despite its importance, few studies, however, have directly examined the ability of nonhuman animals to recall events that span across multiple environments, which is a hallmark of human memory.

The abilities of Lister Hooded rats and Common marmosets to learn and recall overlapping sequences of objects were examined (see Chapters 2 and 3 respectively), with the aim to model memory for events across environments. The sequences relied on contextual cues to determine their correct order, with the added complexity of requiring a shift in sequence if the context changed. Rats were included to assess how the absence of a granular prefrontal cortex influences the capacity to learn and recall complex sequences, and marmosets served as an intermediary model, bridging the gap between rodent studies and larger primate research by providing insights into the role of a simplified granular prefrontal cortex.

Marmosets demonstrated a stronger ability to learn context-dependent sequences compared to rats; however, both species struggled to dynamically adjust their object selection when the contextual environment shifted mid-trial. These findings suggest that while both species can encode context and sequence information, the ability to integrate these dynamically presents significant challenges. This limitation persisted despite the evolutionary advantage of marmosets' more developed brain structures, particularly their granular lateral prefrontal cortex, which is absent in rats (Burman et al., 2006).

The behavioural differences closely align with known anatomical distinctions. The granular lateral prefrontal cortex in marmosets – a feature shared with Old World monkeys such as Rhesus macaques – has been linked to serial order memory, a capability enabling macaques to identify which items appeared earlier in a sequence after a brief delay (Petrides, 1997). Evolutionarily, marmosets are more closely related to macaques than to rats, and the greater prefrontal cortical development observed in macaques reflects a hierarchy of cognitive capacities across these species. Nevertheless, whether the additional prefrontal regions in macaques directly account for their enhanced sequence memory remains an open question, highlighting the need for further comparative studies.

Interestingly, rodents have demonstrated comparable capabilities in tasks involving sequences of odours, offering insights into the role of conserved brain regions in sequence memory. In rats, damage to the hippocampus and medial prefrontal cortex selectively impairs the ability to judge the order of items without affecting object recognition (Fortin et al., 2002; Eichenbaum, 2017). This finding highlights that sequence processing is not solely reliant on evolutionarily advanced brain structures like the granular prefrontal cortex. Instead, it underscores the contribution of older, homologous neural circuits, illustrating the interplay between conserved and specialised mechanisms in supporting sequence memory across species.

This interplay is particularly evident from an evolutionary perspective, as the ability to organise sequences of actions in response to contexts is crucial for goal-directed behaviours. Foraging is a prominent example, as it requires animals to learn and recall structured routes, enabling them to optimise search strategies by remembering the timing and location of food sources across varying contexts such as seasonal changes or daily movement patterns. This adaptability provides evolutionary advantages, enabling animals to adapt, conserve energy, and maximise resources in unpredictable environments.

Building on this evolutionary foundation, studies of contextual memory, particularly in rodents, have provided valuable insights into these mechanisms. Episodic-like memory has shown to be a process likely supported by the medial prefrontal cortex – a region conserved across rats, marmosets and, macaques (see Chapter 1.4). In coordination with the hippocampus – a similarly organised brain area across these

species (see Chapter 1.5) – the medial prefrontal cortex is pivotal in managing the associations within a memory (Fortin et al., 2002; Kesner et al., 2002; DeVito & Eichenbaum, 2011). These conserved neural substrates underpin memory processes across many species, forming a foundational framework for understanding the processes underlying episodic memory in humans. Research in nonhuman primates, however, has often focused on abstract rule-learning tasks that rely on their advanced frontal brain regions, emphasising the need for studies that explore their ability to integrate sequences and contexts in a more ecologically valid manner.

The present study sought to address this gap by assessing macaques' ability to learn context-dependent sequences, building on findings from Chapters 2 and 3, which examined similar tasks in rats and marmosets. These earlier studies revealed challenges in dynamically integrating context and sequence information, highlighting the need for comparative approaches across species. Expanding this research to macaques offers critical insights into the evolutionary and neurobiological bedrock of episodic-like memory. Unlike many prior experiments in macaques, this study incorporated free movement, allowing monkeys to encode spatial contexts in a more dynamic and naturalistic manner. This approach mirrors conditions commonly used in rodent studies, where free movement is essential for understanding the interplay between spatial navigation and context-dependent memory and bridges the gap between primate and rodent research paradigms.

To adapt the task used with rats and marmosets in previous chapters for use with rhesus macaques, modifications were necessary due to their larger size, which made constructing a maze impractical. Instead, the macaques were tested within their home-units, where a touchscreen was mounted on each door for interactive object choice and reward delivery. The background colour of these screens served as the contextual cue, while the animal's physical environment provided an additional physical context, as the colour remained static within each home-unit. This setup leveraged spatial navigation alongside static cues, merging the characteristic of rodent tasks that emphasise movement with primate paradigms that often rely solely on stationary cues.

The study also investigated the role of the prefrontal-hippocampal circuit in episodic memory retrieval, employing non-invasive transcranial ultrasound stimulation. This approach enabled a within-subjects design, avoiding the limitations associated with

irreversible lesions typically employed in such studies. Detailed methods of findings related to this stimulation approach are discussed further in Chapter 5. This chapter, instead, focuses on the training procedures and results from trials involving no stimulation (or "sham" stimulation). These findings will help identify potential biases in baseline behaviour, providing insight into whether the challenges observed relate to context memory, sequence memory, or the integration of the two.

4.2. Methods

4.2.1. Subjects

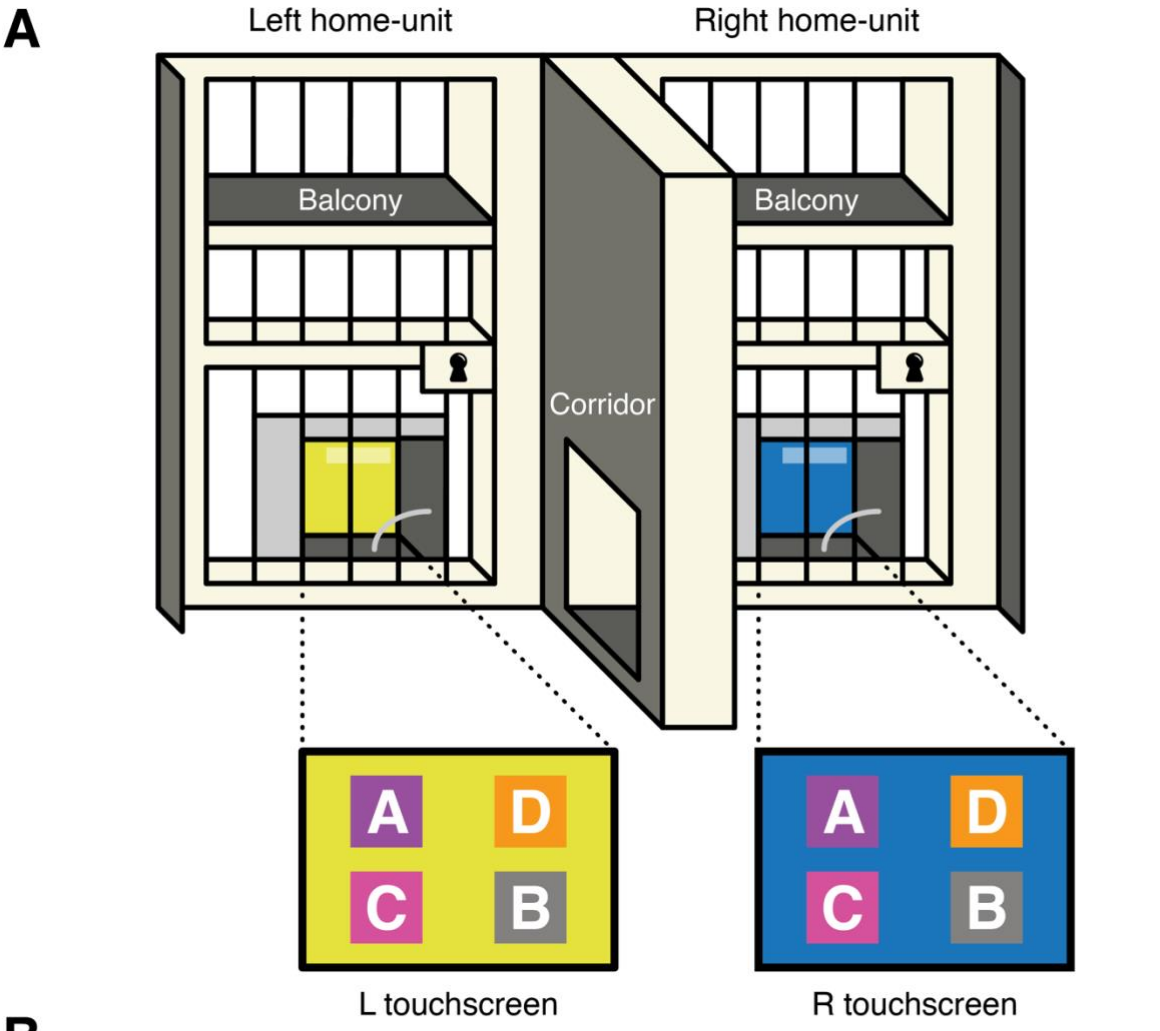
Two rhesus macaques (*Macaca mulatta*), one male (monkey PL: 16 years old and 14 – 15 kg) and one female (monkey MC, 9 years old and 6 – 7 kg) were involved in the experiment. The two participating monkeys were housed separately to each other but resided with another macaque (PL with a male, and MC also with a male) on a daily basis. The colony in which the primates were housed was populated by an average of 40 macaques grouped in twos or threes. Each pair had access to an enclosure divided into two home-units by a central corridor (one home-unit measured L: 2.4 m, W: 1.4 m, H: 2.3 m, Figure 4.1A). The colony had a 12-hour light-dark cycle from 07:00 to 19:00, with temperature (16 – 25 °C) and humidity (40 – 70 %) kept stable. All participating animals were fluid restricted throughout the experiment and testing occurred during the light phase on a consistent schedule (between 09:00 – 13:00). All procedures undertaken were in accordance with the guidelines of the UK Animals (Scientific Procedures) Act of 1986, approved by Newcastle University's Animal Welfare and Ethical Review Body and complied with the European Directive on the protection of animals used in research (2010/63/EU).

4.2.2. Apparatus

Behavioural testing involved the animals interacting with two touchscreen units that were attached to the home-unit as and when required (Figure 4.1A). Both animals had access to the screen through thin bars that ran vertically in front of the screen (Figure 4.1C). A spout was located centrally below the screen and provided a fluid reward (Ribena Blackcurrant Juice, Suntory, Bristol, UK) for every correct response.

4.2.3. Materials

The stimuli onscreen were photographs of real objects described in Chapter 2 (Figure 4.1B). All stimuli images were the same size (200 by 200 pixels) with the objects presented being distinct in their shape and colour presented before a neutral grey wall. The experiment was run through PsychToolbox Version-3 (Brainard, 1997; Pelli, 1997; Kleiner et al., 2007) via MATLAB (version 2017a/2018a) on a Windows machine (Windows 10 Enterprise) connected to the touchscreen.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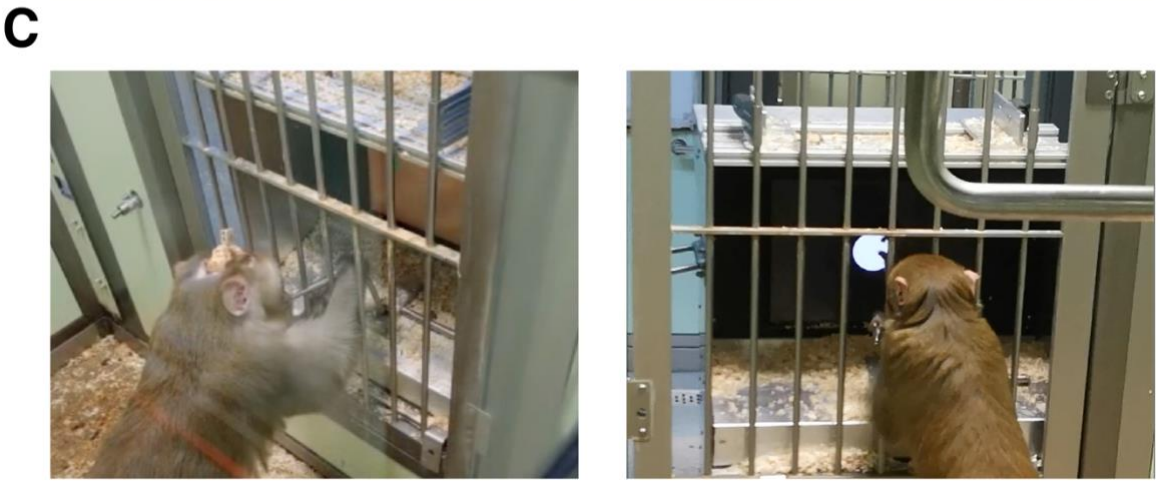
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Figure 4.1. Enclosure setup, touchscreen apparatus and visual stimuli.

A) *Schematic representation of the experimental setup, showing two adjacent home-units connected by a central corridor. Each home-unit was equipped with a touchscreen, located at the base of the door, which the macaques could interact with.* ***B)*** *Visual stimuli presented to the macaques on the touchscreens. Four distinct objects were used: a purple bear (A), a clear saltshaker (B), a pink watering can (C) and an orange spray bottle (D). The labels on the objects are for illustrative purposes and were not visible to the animals during testing.* ***C)*** *Photographs showing the macaques engaging with the touchscreen devices from within their home-units. The animals interacted with the touchscreen through the vertical bars of the enclosure door.*

4.2.4. Design

Before a testing session, the participating macaque was separated from their companion animal and the two touchscreens were attached to the home-units as described above. The two touchscreens were connected with a coaxial cable (BNC, Bayonet Neill-Concelman) attached to LabJack (U3, https://labjack.com) devices present in both machines. This was to allow the touchscreens to work simultaneously on the same task.

An example trial is shown in Figure 4.2. To begin, the animal must touch the white circle that occupies the centre of the screen to initiate the trial. After an inter-stimulus interval (ISI) of 1 second, the stimuli are presented onscreen, and the animal must make a choice to receive a reward. The time taken to make the first touch is recorded (Response 1 RT) and if correct, the animal receives ~ 1.5 ml of juice. If incorrect, the trial is aborted, and the animal is presented with a white spot to initiate the next trial. If correct, however, the animal has the opportunity to make a second choice. The transition between the first and second choice is called the inter-response interval (IRI). If the second choice is correct, the animal will receive another juice reward (~ 1.5 ml) and the time taken to touch the screen (Response 2 RT) will be recorded. Then, after an inter-trial interval (ITI) of 1 second, the next trial will become available to start. If the choice made is incorrect, the trial will abort without delivering a juice reward and the next trial will become available.

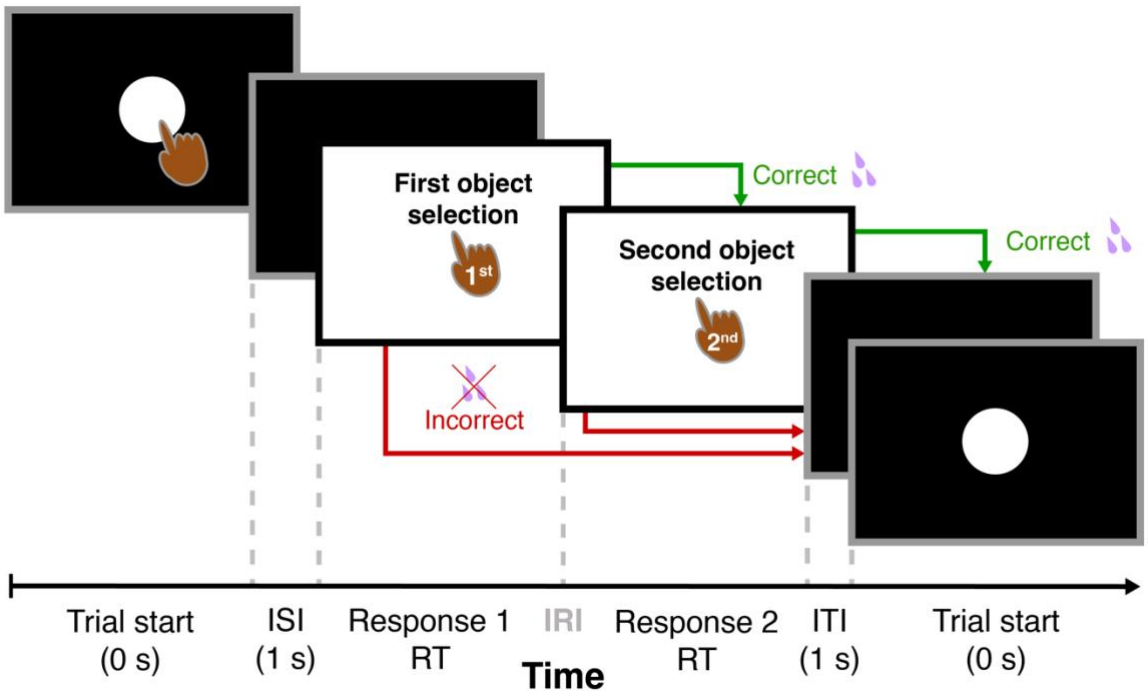

Figure 4.2. Trial sequence and outcomes during an example trial.

The timeline illustrates the progression of a single trial in the touchscreen-based task. The trial begins with the appearance of a central white circle, which the macaque must touch to initiate the trial. After the inter-stimulus interval (ISI, 1 s), four objects appear on the screen. The macaque makes the first selection, which is recorded as Response 1 (RT: reaction time). If the selected object is correct (green arrow), the trial proceeds to the second object selection and the animal receives juice. If the selection is incorrect (red arrow), the trial ends prematurely, followed by an inter-trial interval (ITI, 1 s). Following a short inter-response interval (IRI), the macaque selects a second object (Response 2 RT). If the second choice is correct, the trial ends with juice (green arrow), followed by the ITI. If the second selection is incorrect, the trial terminates without juice (red arrow). The trial concludes with an ITI (1 s) before the next trial begins.

4.2.5. Habituation Procedure

To familiarise the animals with the touchscreens and associating object selection with a reward, each animal underwent two sessions (each comprising of a two-hour block on a given day). During these sessions, the two objects were presented individually, in the order relevant to the task (Figure 4.3A). This setup ensured there were no incorrect trials, as the objects remained on the screen until pressed.

4.2.6. Training Procedure

4.2.6.1. Basic Training: Spatial Contexts

Following habituation, the primates underwent training on the memory task in progressive stages of increasing difficulty. During training, only one screen was attached to the home-unit during a session to minimise distractions. The same touchscreen was consistently assigned to the same side of the home-unit across sessions. In the initial training stage, spatially fixed contexts were used to help the primates associate object sequences with specific contexts. The left touchscreen exclusively presented trials involving a Yellow context, while the touchscreen on the animal's right displayed trials relating only to the Blue context.

Furthermore, training on each context type was counterbalanced across sessions to prevent a learning bias towards one sequence. In the initial training phase, pairs of objects were presented simultaneously in fixed positions on the screen during each trial (Figure 4.3B). For trials in the Yellow context, object A always occupied the top-left quadrant, and object B the bottom-right quadrant. In the Blue context, object C was positioned in the bottom-left quadrant, and object D in the top-right quadrant. This arrangement helped teach the animals the importance of object identity and sequential order within each context. As such, a trial could be categorised as correct (if both choices were correct), half-correct (if the first choice was correct but the second choice was incorrect), or incorrect (if the first choice itself was incorrect). If the animal made an incorrect choice at any point during the sequence, the trial was aborted, and the animal had to wait a delay of 1 secs before starting the next trial.

4.2.6.2. Intermediate Training: Object Identity vs. Location

After several sessions with the spatially-fixed object training trials, the animals were introduced to trials in which the objects were presented simultaneously but could now appear in the opposite location than they were previously accustomed to (Figure 4.3C). This adjustment aimed to teach each animal to prioritise object identity over spatial location. For example, in Yellow context trials, objects A and B could appear in either the top-left or bottom-right corners, while in Blue context trials, objects C and D could occupy the opposite diagonal axis, appearing in either the bottom-left or top-right quadrants.

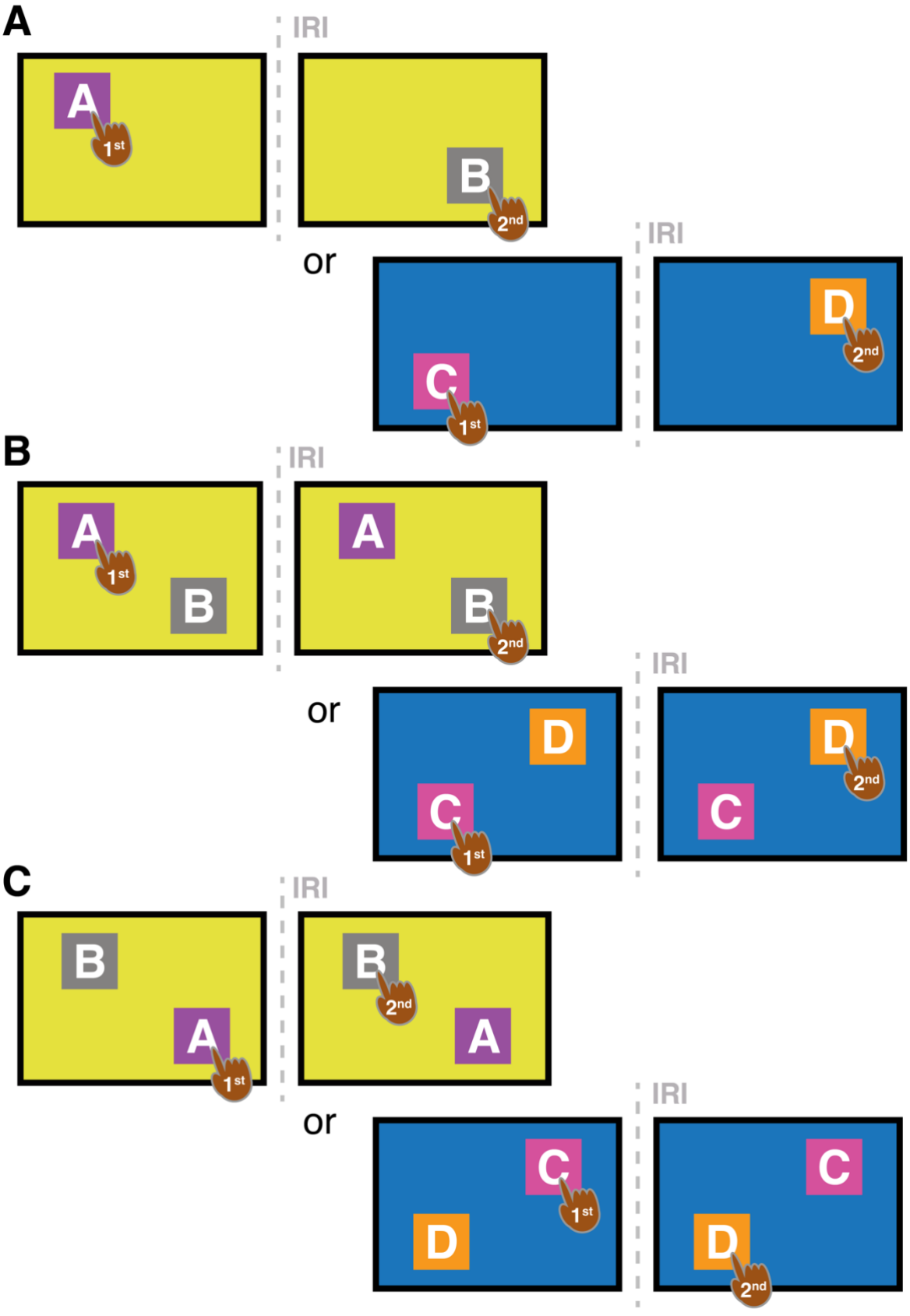
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Figure 4.3. Schematic representation of habituation and training trials.

A) *Depicts the initial trials in which objects were presented individually, allowing each animal to become familiar with selecting objects in a sequence. This phase was designed to encourage interaction with the touchscreen and establish a basic understanding of the task.* ***B)*** *In the next phase, objects were displayed together, but their spatial positions remained fixed. This was intended to further reinforce object selection while introducing the concept of making choices from multiple options.* ***C)*** *The final phase involved reversing the spatial locations of the objects, emphasising the importance of object identity over spatial position. This phase trained the animals to focus on the objects themselves rather than relying on their fixed locations.*

Due to difficulty in learning the correct sequential order of objects in each context, monkey MC received additional training steps to reinforce the importance of sequence over spatial location. This involved a further three sets of training trials. In the first, the initial object appeared 0.5 seconds prior to the second object onscreen (Figure 4.4A). In the second, the first object remained in the same location for all trials, while the second object could appear adjacent, diagonally opposite, or directly above or below the first object (Figure 4.4B). In the final subset, the first object was fixed in one quadrant, while the second appeared above, below, or to its right, but never to its left. (Figure 4.4C). This was to ensure the animal did not have to focus on sequential movement and more on object identity.

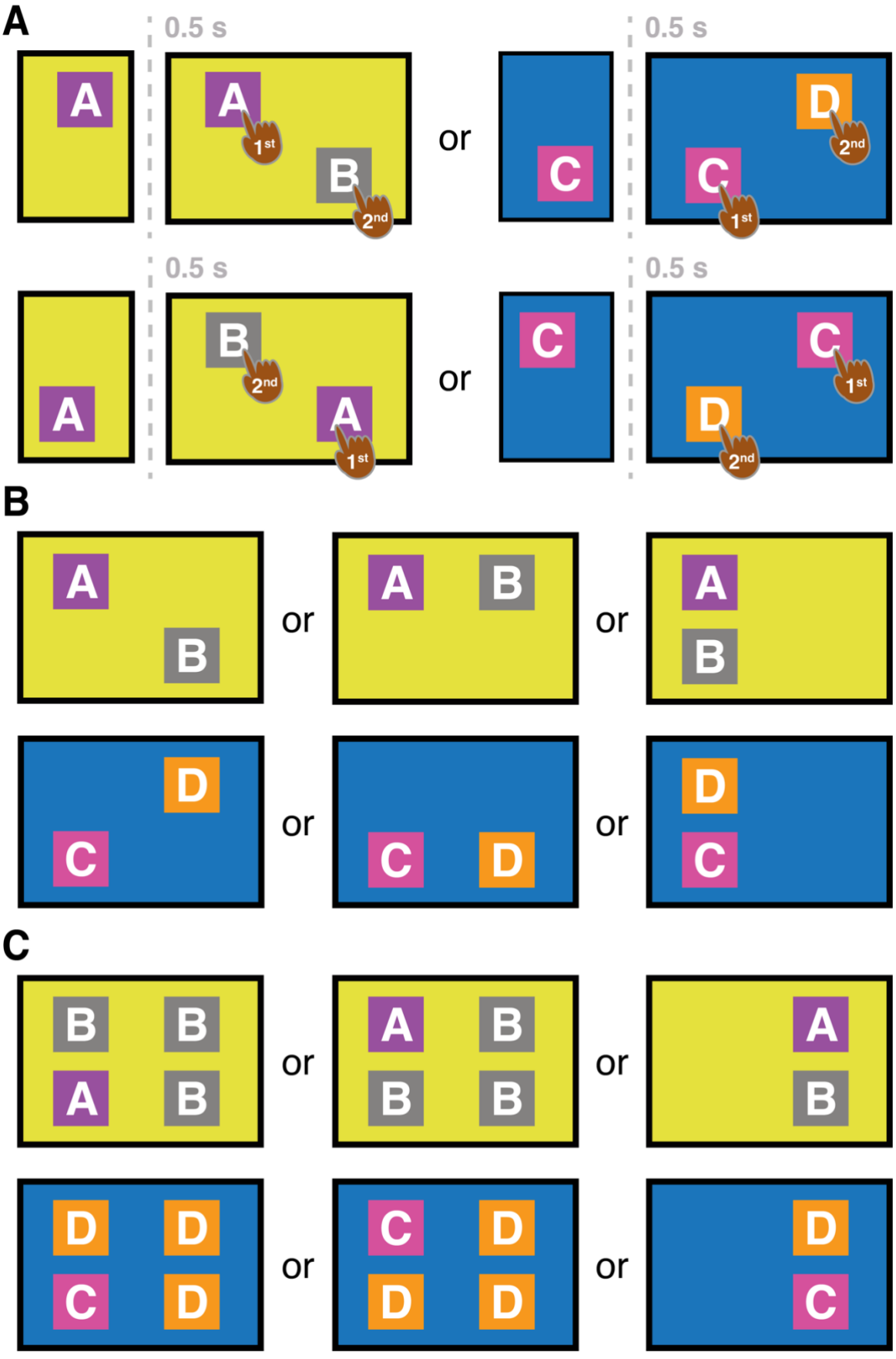
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Figure 4.4. Additional training steps to reinforce sequence learning in monkey MC.

A) *Sequence discrimination with temporal overlap: The first object appears onscreen, followed 0.5 seconds later by the second object. The monkey is required to identify the temporal order of objects depending on background context.* ***B)*** *Sequence reinforcement with fixed first-object location: the first object remains in the same spatial location across trials, while the second object appears adjacent, diagonally opposite, or directly above or below the first object.* ***C)*** *Generalised sequence reinforcement: the first object was fixed in one quadrant, while the second appeared above, below, or to its right, but never to its left.*

4.2.6.3. Incongruent Objects and Contextual Backgrounds

In the next phase of training, all four objects were presented on-screen during a single trial. This step aimed to introduce the concept of incongruent objects and teach the animals that they needed to ignore certain objects based on the background colour of the screen. For example, when the background was Yellow, the target objects (congruent) were objects A and B, while the objects to be avoided (incongruent) were objects C and D (Figure 4.5A). Conversely, when the background colour was blue, objects C and D became the target objects, and A and B were the ones to be ignored (Figure 4.5B). To introduce this concept without overwhelming the animals, the incongruent objects remained static across trials, while the positions of the congruent objects could switch places with one another.

The final phase of training emphasised the fact that the spatial location of both the congruent and incongruent objects was irrelevant. This phase included 24 possible trial permutations which were categorised into six distinct categories described below.

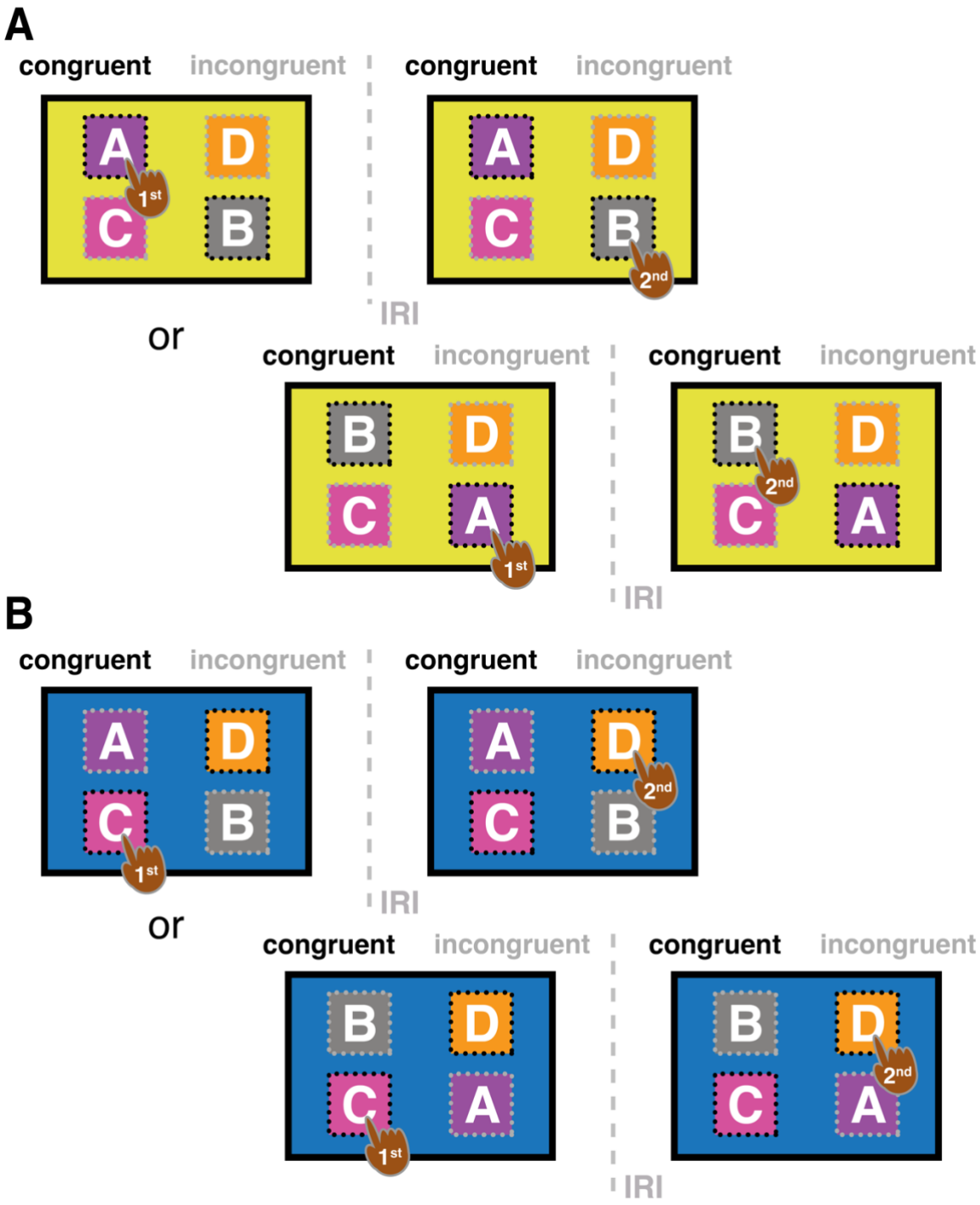

Figure 4.5. Training phase introducing congruent and incongruent objects.

A) *With a yellow background, objects A and B served as the target (congruent) objects, while objects C and D were incongruent and to be ignored.* ***B)*** *With a blue background, objects C and D became the target (congruent) objects, while objects A and B were incongruent. During training, the incongruent objects remained in fixed positions across trials, while the congruent objects could change positions. Black dots represent congruent objects, and grey dots represent incongruent objects. IRI refers to inter-response-interval.*

4.2.6.4. Sequence Learning with Context Switching

In the initial training discussed earlier, the congruent objects for each context consistently occupied the same diagonal space across trials. Specifically, in the Yellow context, object A always occupied the top-left quadrant, while object B was located in the bottom-right quadrant. In the Blue context, object C was always present in the bottom-left quadrant, and object D was positioned in the top-right quadrant.

In the latter training stage, trials where the congruent objects maintained these same positions were classified as ‘Familiar’ trials (Figure 4.6A). Conversely, ‘Reverse’ trials featured the congruent objects in the opposite positions compared to those seen in the ‘Familiar’ trials (Figure 4.6B). For the ‘1stBlue’ (Figure 4.6C) and ‘1stYellow’ (Figure 4.6D) trials, only the first object in each sequence appeared in its familiar position. In the ‘1stBoth’ trials (Figure 4.6E), both sequences had their first object in familiar positions. Finally, in ‘2ndBoth’ trials (Figure 4.6F), only the second objects in both sequences occupied their relevant spatial locations.

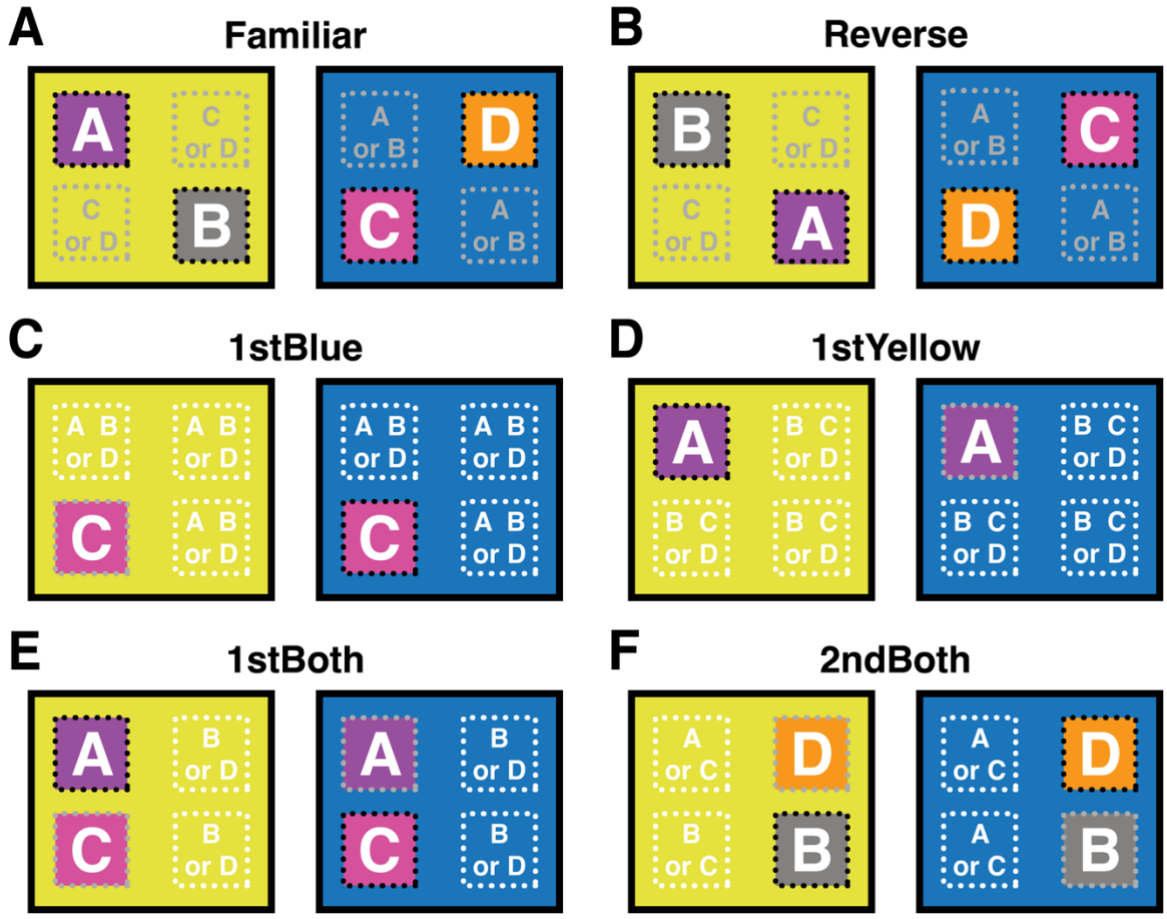

Figure 4.6. Schematic representation of the six trial types.

A) *Familiar: The congruent objects maintain their original spatial positions (A and B for Yellow context, C and D for Blue context).* ***B)*** *Reverse: The congruent objects switch to the opposite spatial positions.* ***C)*** *1stBlue: Only the first object in the sequence is in its familiar position for the Blue context.* ***D)*** *1stYellow: Only the first object in the sequence is in its familiar position for the Yellow context.* ***E)*** *1stBoth: The first object in both contexts occupies its familiar position, while the second object is repositioned.* ***F)*** *2ndBoth: Only the second object in the sequence appears in its original spatial position for both contexts. These trial types were designed to progressively teach sequence flexibility by varying spatial and contextual cues.*

4.2.7. Testing Procedure

4.2.7.1. Stable Context Testing

For the testing procedure, two touchscreens were attached to the home-units, as illustrated in Figure 4.7. Similar to the training phase, the initial testing stage limited individual contexts to one side of the home-unit (denoted as ‘stable context’, with Yellow context trials on the left and Blue context trials on the right, Figure 4.7A). To

ensure the animals engaged with an equal number of each type of trial, the touchscreens were programmed to switch off after five trials, regardless of the number of correct responses. This setup required the animals to actively move between the screens to continue the task.

4.2.7.2. Context Reversal Testing

Next, the animals were tested on their ability to adapt to change. The screens and corresponding background colour remained stable, with the Yellow context on the left and Blue context on the right; however, the relevant object sequences were switched (Figure 4.7B). Consequently, the animals had to learn that for the Yellow context, objects C and D, rather than A and B, were the correct choices, and they needed to select them in that order. Conversely, for the Blue context, objects A and B became the congruent objects. Only monkey PL participated in this phase due to difficulties in participation with monkey MC.

4.2.7.3. Unstable Context Testing

The final testing stage involved removing a stable form of context spatially, with both Yellow and Blue context trials appearing randomly on either the left or right-side home-unit touchscreen. In addition, to test each animal's understanding of context, novel trials involving a contextual change mid-sequence were introduced (Figure 4.7C). In these new trials, after the first object was selected, the background colour would either change to the opposite colour or remain the same colour as previously was onscreen. If it changed to the former, the animal needed to update its sequence mid-trial and respond to the new context by selecting the object that corresponded to the second element of the sequence relevant to that new context. For example, if the trial began with a yellow background and changed mid-way to a blue background, the animal would need to first choose object C for the Yellow context and then object B for the Blue context (Figure 4.7C).

Due to reduced motivation during testing, monkey MC was tested on only one touchscreen per session, with the side of the home-unit to which it was attached counterbalanced across sessions. In contrast, monkey PL continued to switch between screens every five trials. Trials for monkey PL were conducted either in the Yellow home-unit (previously associated only with Yellow context trials, on the animal's left) or the Blue home-unit (previously associated only with Blue context trials,

on the animal's right). Monkey MC, however, performed the entire session either in the Yellow home-unit or in an adjacent 'unfamiliar' home-unit, which had not been used for training or testing at any point and thus was not linked to any specific context.

4.2.8. Behavioural Analysis

This task was performed as part of the transcranial ultrasound stimulation (TUS) experiment outlined in Chapter 5. To quickly summarise, this involved localised bursts of ultrasound being applied to one of several studied brain areas to examine the effects of TUS on context-dependent sequence comprehension. For both macaques, the stimulated brain area was counterbalanced across sessions, which also included several 'sham' stimulation sessions. During these sham sessions, the animals underwent the same procedures as the sonication sessions but without any ultrasound being applied. As such, to avoid potential conflicts and confounds from sessions where the animals were sonicated, the following analyses focus solely on trials where no stimulation was applied, and henceforth, referred to as sham trials.

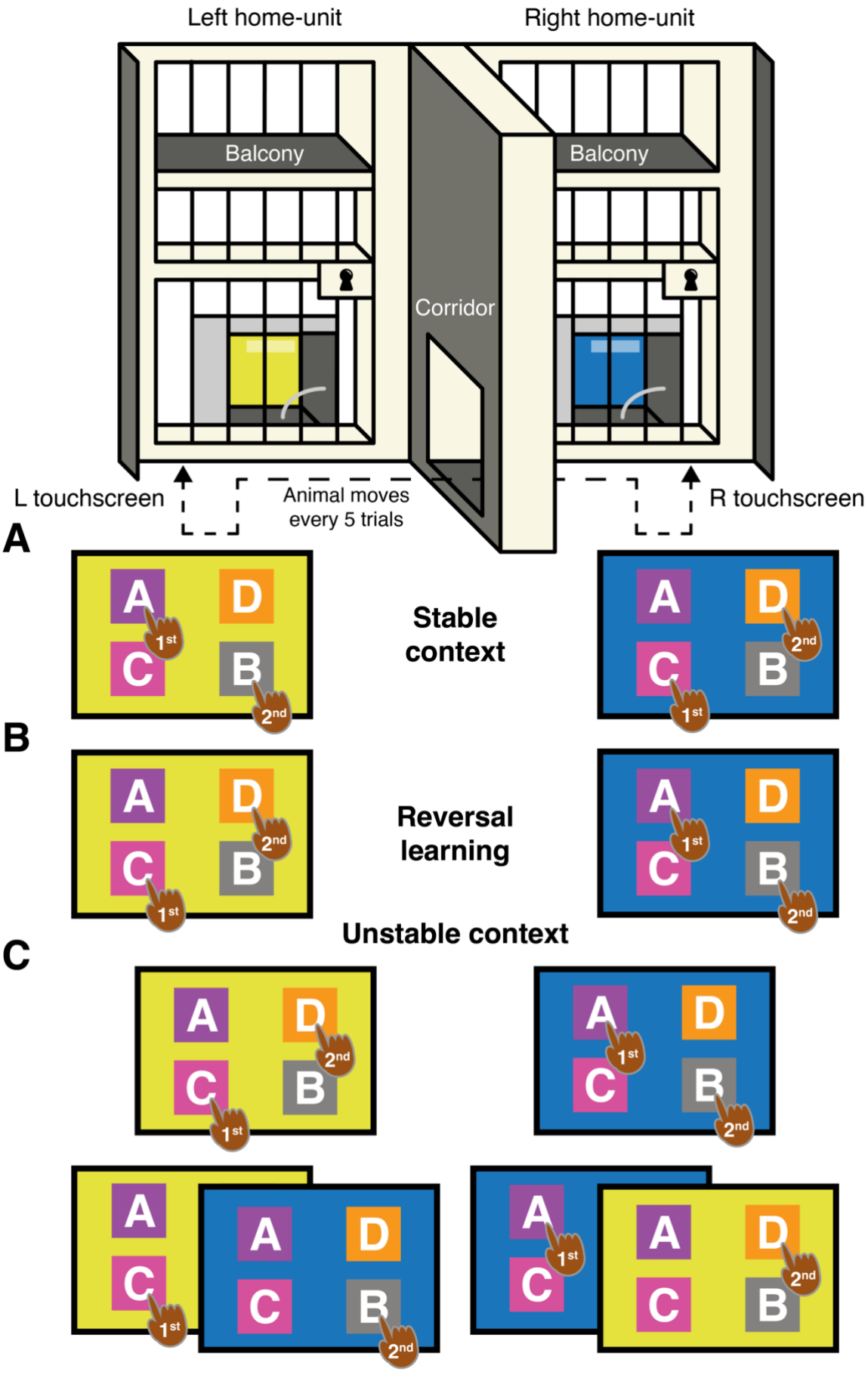
Left home-unit
Right home-unit
Balcony
Balcony
Corridor
L touchscreen
Animal moves
every 5 trials
R touchscreen
A
Stable
context
B
Reversal
learning
Unstable context
C
A
D
C
B
1st
2nd

Figure 4.7. Testing procedure phases illustrating context and task variations.

A) *Stable context: Yellow context trials occur on the left touchscreen, and Blue context trials on the right touchscreen, with animals required to alternate between the two every five trials.* ***B)*** *Reversal learning: Contexts remain spatially fixed, but the congruent object sequences are reversed (objects A and B for Blue context, objects C and D for Yellow context).* ***C)*** *Unstable context: Contexts are randomly presented on either touchscreen. Trials include mid-sequence contextual changes requiring the animal to update the sequence based on the new background colour.*

4.3. Results

4.3.1. Stable context

To evaluate individual performance during the stable context phase, error ratios were calculated for each macaque. Error ratios were defined as the number of errors made divided by the total number of trials attempted during a session. Given that each trial required the selection of two objects in a specific order, three distinct error types could occur: '1st Object' errors, '1st Object Repeated' errors, and '2nd Object' errors.

1st Object errors occurred when the initial object selected was incorrect. 1st Object Repeated errors were defined as trials in which the first object was selected correctly, but the same object was incorrectly chosen again as the second selection. 2nd Object errors referred to incorrect selection of the second object, provided the first selection was correct and distinct.

It is important to note that if the 1st object was incorrectly chosen, the trial was coded as incorrect and aborted, and no second object selection was made. Therefore, any trials classified as '2nd Object errors' were by definition '1st Object correct' trials, in which the first object was selected correctly, but the second object was incorrectly chosen.

To assess differences in error distribution, a linear mixed-effects model was conducted with error type (1st Object, 1st Object Repeated, 2nd Object) and context (Yellow, Blue) as fixed effects, including their interaction. Macaque identity was included as a random intercept to account for inter-individual variability.

The model revealed a significant main effect of error type (F (2, 125) = 16.8, p < .001), indicating that certain types of errors were more frequent than others. Post-hoc comparisons (with Tukey correction) showed that 2nd Object errors occurred significantly less frequently than 1st Object errors (t (125) = - 4.51, p < .001, d = 0.96). In contrast, 1st Object Repeated errors were significantly more frequent than 2nd Object errors (t (125) = 5.40, p < .001, d = 1.15), suggesting a notable tendency to perseverate on the initial choice (Figure 4.8A).

Interestingly, there was no significant difference in frequency between 1st Object errors and 1st Object Repeated errors (t (125) = - 0.887, p = 0.650, d = - 0.189), indicating that errors at the initial and repeated selection stages were similarly likely, potentially

reflecting occasional lapses in attention or a tendency for perseverative responses within a static environment, rather than specific difficulties with task rules.

There was no significant main effect of context (F (1, 125) = 0.237, p = 0.627), nor a significant interaction between context and error type (F (2, 125) = 0.714, p = 0.492, Figure 4.8B), suggesting that the stable visual background did not influence error distribution. This consistency across contexts implies that, under stable environmental conditions, error types were robust and predictable.

Overall, the reduction in 2nd Object errors suggests that macaques were generally able to maintain object sequence knowledge once initiated, and that stable contexts facilitated performance by reducing external distraction. These findings further highlight the stabilising role of an unchanging context in promoting consistent performance.

4.3.2. Reversal Phase

The Reversal phase required monkey PL to adapt to changes in object-context associations while the contextual cues (screen position and background colour) remained stable. Specifically, the previously learned object sequences were swapped between contexts – for example, the object A to object B sequence formerly associated with the Yellow context, was now assigned to the Blue context, and vice versa (Figure 4.7B). This manipulation necessitated relearning familiar object sequences under altered contextual conditions.

To assess how error patterns changed during relearning, a linear mixed-effects model was fitted with error type (1st Object, 1st Object Repeated, 2nd Object) and context (Yellow, Blue) as fixed effects, along with their interaction. Session date was included as a random intercept to account for between-session variability.

The model revealed a significant main effect of error type (F (2, 20) = 6.01, p = 0.009), indicating that certain errors occurred more frequently than others (Figure 4.8C). In contrast, there was no significant main effect of context (F (1, 20) = 1.34, p = 0.261), nor a significant interaction between error type and context (F (2, 20) = 0.216, p = 0.808, Figure 4.8D). These results suggest that although monkey PL exhibited differential error patterns across trial types, these patterns were not modulated by contextual cues. This implies that performance deficits were not driven by the colour-

coded contexts per se, but rather by difficulty in adapting to the reversal of object-context associations.

Post-hoc comparisons confirmed that 1st Object errors were significantly more frequent than both 2nd Object errors (t (20) = 2.92, p = 0.022, d = 1.31) and 1st Object Repeated errors (t (20) = 3.08, p = 0.016, d = 1.38), indicating a particular vulnerability at the initial stage of sequence retrieval. However, there was no significant difference between 2nd Object and 1st Object Repeated errors (t (20) = 0.156, p = 0.987, d = 0.070), suggesting similar rates of perseverative versus sequential completion errors (Figure 4.8C).

The elevated incidence of 1st Object errors likely reflects a disruption in the initial retrieval of the object sequence, possibly driven by interference from prior associations that were no longer valid. The fact that error rates declined once the first object was correctly selected implies a relatively preserved memory for object-to-object transitions within the sequence, despite weakened object-context links. This pattern is consistent with the idea that the reversal manipulation primarily impaired contextual retrieval cues, rather than the internal structure of the object sequences themselves. The stability of context-specific error types across Yellow and Blue backgrounds further supports the interpretation that context rule switching – rather than contextual ambiguity – was the primary source of interference during relearning.

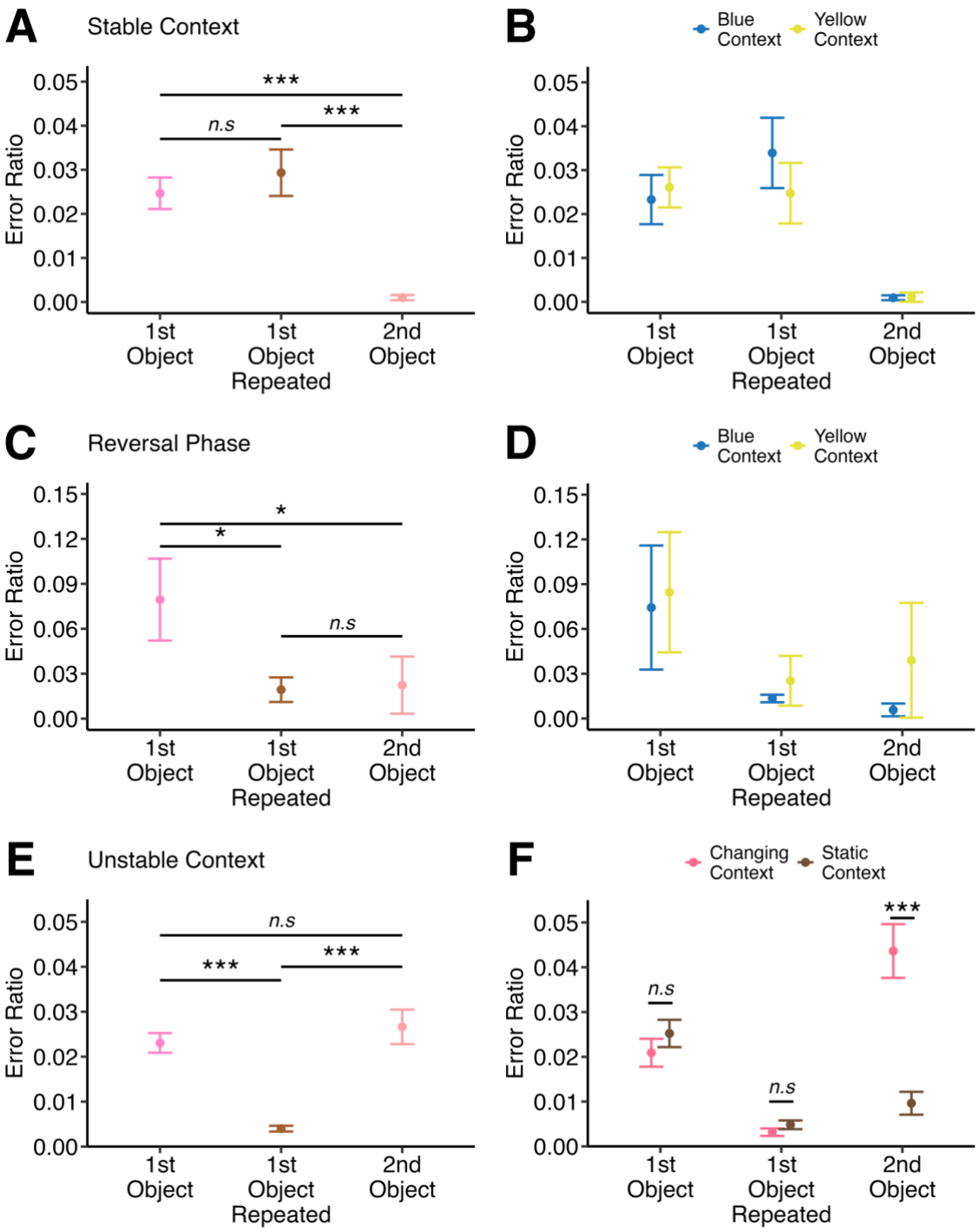

Figure 4.8. Error ratios for object selection during stable, reversal, and unstable phases, split by error type and context.

A) *Error ratios in the stable context condition for three error types: '1st Object', where the first object chosen was incorrect; '1st Object Repeated', where the first object was incorrectly chosen for both sequence elements; and '2nd Object', where the second object chosen was incorrect.* ***B)*** *Error ratios in the stable context condition, separated by Blue and Yellow contexts, across the same error types.* ***C)*** *Error ratios during the reversal phase for the three error types.* ***D)*** *Reversal phase error ratios split by Blue*

and Yellow contexts. ***E)*** *Error ratios in the unstable context condition for the three error types.* ***F)*** *Error ratios in the unstable context condition, separated into changing and static contexts. Dots represent the mean error ratio, and error bars indicate ± SE. Significance is indicated by *** (p < .001), * (p < .05) or n.s (p > .05).*

4.3.3. Unstable context

The unstable context phase was designed to test the macaques' flexibility in responding to dynamic contextual changes and their ability to adapt when stable spatial physical cues were removed. Unlike previous phases, the Yellow and Blue context trials were now presented randomly on either the left or right side of the home-unit, eliminating the predictability of spatial context. Additionally, novel trials introduced a mid-sequence contextual shift, requiring the macaques to adapt to a new context mid-trial. For example, after selecting the first object in a sequence associated with one context (e.g., Yellow), the background colour could switch to the opposite context (e.g., Blue), requiring the animal to update its sequence and select the object corresponding to the second element of the new context's relevant sequence (Figure 4.7C). These changes placed increased demands on the macaques' ability to integrate contextual cues dynamically while maintaining correct object-sequence associations.

To investigate how error patterns changed with context instability, a linear mixed-effects model was fitted with error type (1st Object, 1st Object Repeated, 2nd Object) and context change (Static vs. Changing) as fixed effects, along with their interaction. Individual macaque was included as a random intercept to account for inter-subject variability.

The model revealed a significant main effect of error type (F (2, 197) = 28.2, p < .001), indicating significant differences in the frequency of specific error types (Figure 4.8E). Post-hoc comparisons highlighted that 1st Object Repeated errors – reflective or perseveration – occurred significantly less frequently than both 1st Object errors (t (197) = - 5.88, p < .001, d = - 1.01) and 2nd Object errors (t (197) = - 6.98, p < .001, d = - 1.20). However, there was no significant difference between 1st and 2nd Object error rate (t (197) = - 1.10, p = 0.515, d = - 0.190), suggesting a general difficulty in selecting the correct object in accordance with the active context, while perseverative responses were relatively rare.

Critically, the model also identified a significant main effect of context change (F (1, 197) = 12.5, p < .001), demonstrating that overall error rates increased under conditions of dynamic context switching (Figure 4.8F). Post-hoc analysis confirmed that both macaques made significantly more errors during trials where the context changed mid-sequence compared to trials with a static context (t (197) = 3.53, p < .001, d = 0.494), supporting the hypothesis that mid-trial shifts in contextual information impose additional cognitive load.

Furthermore, a significant interaction between error type and context condition (F (2,197) = 21.8, p < .001) indicated that the specific nature of errors was differentially affected by whether the context transitioned or remained stable within the trial. Post-hoc comparisons revealed that 1st Object errors (t (197) = - 0.939, p = 0.349, d = - 0.228) and 1st Object Repeated errors (t (197) = - 0.361, p = 0.719, d = - 0.088) were unaffected by contextual stability. In contrast, 2nd Object errors were significantly more frequent in trials with a changing context (t (197) = 7.41, p < .001, d = 1.80, Figure 4.8F), indicating a pronounced difficulty in sequence updating following a mid-trial contextual shift.

Taken together, these results suggest that while the macaques were generally capable of initiating context-appropriate sequences, they were significantly impaired in continuing those sequences when the context changed partway through the trial. This highlights a specific vulnerability in updating behaviour based on new contextual information, underscoring the cognitive demands of maintaining and modifying hierarchical object-context associations under dynamic environmental conditions.

4.3.4. Comparison of error ratios for stable context sequences

For a more in depth analysis of the types of errors made, errors were categorised into three distinct types: ‘Order’ errors, where the animal selected an object that was relevant to the current context but not in the correct sequential order; ‘Context’ errors, where the animal chose an object that adhered to the correct sequence order but belonged to the opposite context; and ‘Full’ errors, where both the selected object and its sequence were incorrect for the given context. The specificities of these errors are described further in Table 4.1 for the Stable context phases, and Table 4.2 for the sequences seen in the Reversal and Unstable Context phases.

Table 4.1. Error types for cognitive errors for the stable phase.

Context	Object	Choice Position	
		1st	2nd
Yellow (Y)	A	+	Order
	B	Full	+
	C	Context	Full
	D	Order	Context
Blue (B)	A	Context	Full
	B	Order	Context
	C	+	Order
	D	Full	+

Abbreviations: + denotes a correct response.

To assess differences in error distribution, a linear mixed-effects model was conducted with error type (Order, Context, Full) and context (Yellow, Blue) as fixed effects, including their interaction. Macaque identity was included as a random intercept to account for inter-individual variability.

The model identified an effect of error type (F (2, 125) = 56.7, p < .001, Figure 4.9A) but not an effect of context (F (1, 125) = 0.213, p = 0.645), nor an interaction between error type and context (F (2, 125) = 0.021, p = 0.979, Figure 4.9B). Post-hoc comparisons highlighted a difficulty in remembering the sequence of objects for each context, with Order errors being made significantly more frequently than both Context errors (t (125) = 9.05, p < .001, d = 1.92and Full errors (t (125) = 9.38, p < .001, d = 2.00, Figure 4.9A). The difference in frequency between Context errors and Full errors was not found to be significant (t (125) = 0.326, p = 0.943, d = 0.070, Figure 4.9A) suggesting that difficulty was in remembering the temporal order of the objects compared to remembering which objects were pertinent to which context.

4.3.5. Comparison of error ratios for reversal learning sequences

Due to logistical problems outlined in the methods (Section 4.2.7.2) , only one monkey (monkey PL) performed the reversal learning phase of the experiment, where the correct sequence of objects was switched between the two contexts. Error rates were again re-categorised to assess the importance of context and sequence individually. The error types and their corresponding trials can be found in Table 4.2 below.

Table 4.2. Error types for cognitive errors for the reversal and unstable phases.

Context	Object	Choice Position	
		1st	2nd
Yellow (Y)	A	Context	Full
	B	Full	Context
	C	+	Order
	D	Order	+
Blue (B)	A	+	Order
	B	Order	+
	C	Context	Full
	D	Full	Context
Yellow-Blue (Y-B)	A	Context	Order
	B	Full	+
	C	+	Full
	D	Order	Context
Blue-Yellow (B-Y)	A	+	Full
	B	Order	Context
	C	Context	Order
	D	Full	+

Abbreviations: + denotes a correct response.

To further examine the nature of errors during the reversal learning phase, a linear mixed-effects model was fitted with error type (Order, Context, Full) and context (Yellow, Blue) as fixed effects, along with their interaction. Session date was included as a random intercept to account for between-session variability.

The model revealed no significant main effects for error type (F (2, 20) = 1.76, p = 0.197, Figure 4.9C) or context (F (1, 20) = 0.893, p = 0.356), and no interaction between the two factors (F (2, 20) = 0.040, p = 0.961(Figure 4.9D). These findings

suggest that monkey PL's performance during the reversal phase was not disproportionately affected by any one type of error, nor was it modulated by the contextual setting.

Descriptively, Order errors were the most common (mean: 0.06 ± SE: 0.03) followed by Context errors (mean: 0.05 ± SE: 0.03) and Full errors (mean: 0.02 ± SE: 0.03). However, these differences did not reach statistical significance, indicating that monkey PL exhibited a relatively uniform error distribution across different error types and contexts during relearning.

This uniformity in error frequency may reflect a generalised difficulty in re-establishing object-sequence-context mappings rather than a specific disruption in a distinct cognitive component. The absence of context-dependent effects further suggests that the errors were not driven by an inability to retrieve or integrate contextual cues per se, but rather by the challenge of reversing previously consolidated associations.

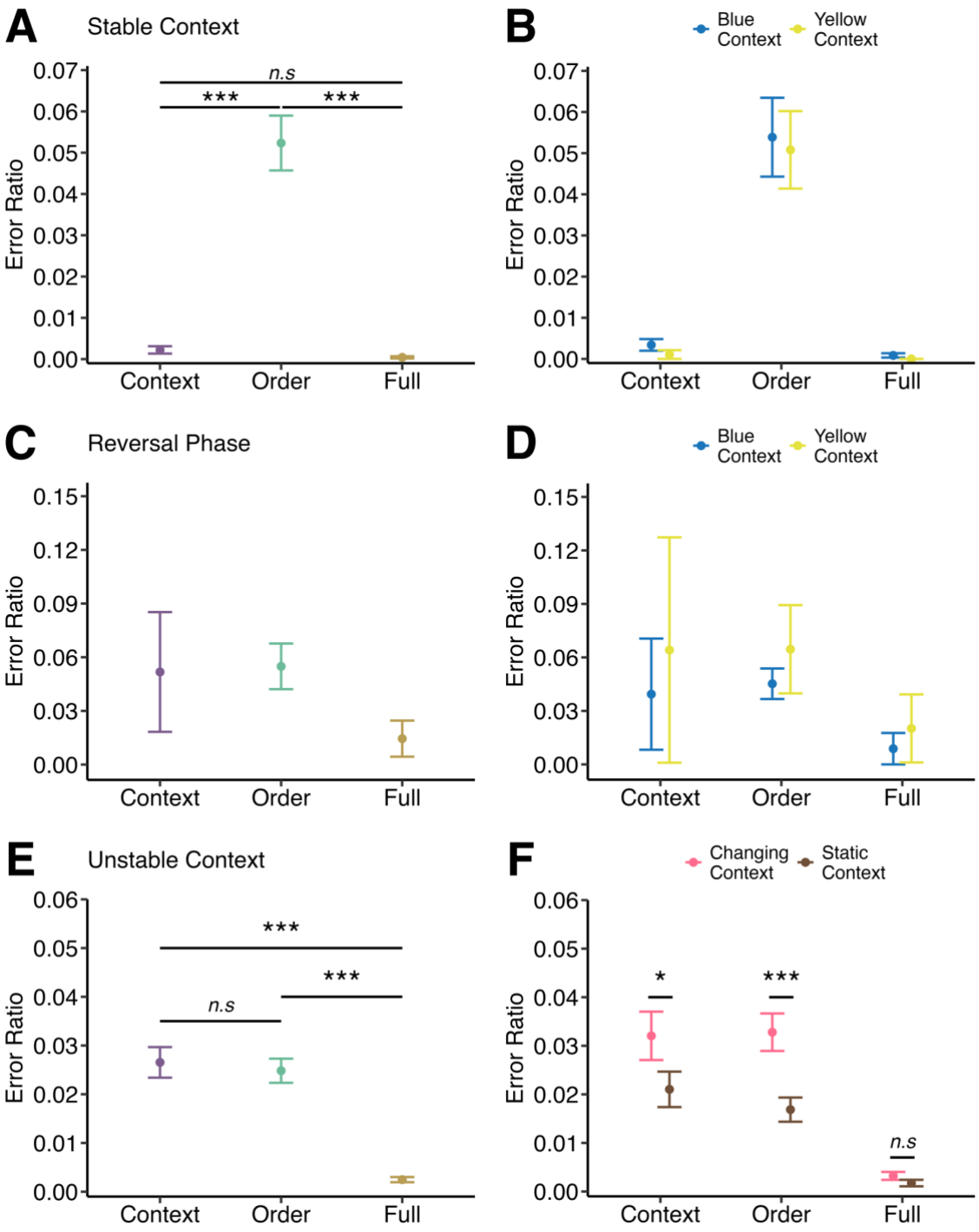

Figure 4.9. Error ratios for object selection during stable, reversal, and unstable phases, split by recategorised error type and context.

A) *Error ratios in the stable context condition for three recategorised error types: 'Order' errors (selection of an object relevant to the current context but in the wrong sequential order), 'Context' errors (selection of an object that followed the correct sequence order but was in the wrong context), and 'Full' errors (selection of an object that was incorrect in both sequence order and context).* ***B)*** *Error ratios in the stable context condition, separated by Blue and Yellow contexts, across the same error*

types. ***C)*** *Error ratios during the reversal phase for the three error types.* ***D)*** *Reversal phase error ratios split by Blue and Yellow contexts.* ***E)*** *Error ratios in the unstable context condition for the three error types.* ***F)*** *Error ratios in the unstable context condition, separated into changing and static contexts. Dots represent the mean error ratio, and error bars indicate ± SE. Significance is indicated by *** ($p < .001$), * ($p < .05$) or n.s ($p > .05$).*

4.3.6. Comparison of error ratios for unstable context sequences

Both monkeys participated in the unstable context phase, during which trials from the Yellow and Blue contexts could be presented randomly in either the left or right home-unit. This spatial unpredictability was further compounded by the introduction of novel trial types involving mid-sequences contextual shifts, whereby the contextual background changed partway through a trial. To analyse performance under these conditions, error rates were categorised into three types – Order, Context, and Full errors – as outlined in Table 4.2.

To assess how error patterns were affected by context instability, a linear mixed-effects model was fitted with error type (Order, Context, Full errors) and context change (Static vs. Changing) as fixed effects, along with their interaction. Individual macaque was included as a random intercept to account for inter-subject variability.

The model revealed significant main effects of error type (F (2, 197) = 35.8, $p < .001$) and contextual change (F (1, 197) = 13.4, $p < .001$), but no significant interaction between these factors (F (2, 197) = 2.69, $p = 0.071$). This indicates that error frequency was independently influenced by both the nature of the error and the stability of the context, without an interaction between the two.

Post-hoc comparison of error types revealed that Order and Context errors occurred at statistically comparable rates (t (197) = - 0.540, $p = 0.852$, d = - 0.09, Figure 4.9E). In contrast, Full errors were significantly less frequent than both Context errors (t (197) = - 7.59, $p < .001$, d = - 1.30) and Order errors (t (197) = - 7.05, $p < .001$, d = - 1.21). These results suggest that the macaques rarely made compound errors that involved both incorrect sequencing and context misidentification. Instead, errors were more likely to stem from failures in either temporal order or contextual discrimination alone.

Analysis of contextual change effects further supported this dissociation. Order errors (t (197) = 3.55, p < .001, d = 0.892) and Context errors (t (197) = 2.46, p = 0.015, d = 0.596) were both significantly more frequent in trials involving a context switch than in trials where context remained static (Figure 4.9F). Full errors, however, were not significantly modulated by contextual change (t (197) = 0.329, p = 0.742, d = 0.080).

These findings highlight the cognitive demands imposed by mid-sequence context changes, which disrupted the macaques' ability to correctly integrate object-sequence information with context cues. However, the relative infrequency of Full errors implies that although performance was challenged, the animals did not completely lose track of both dimensions simultaneously. Rather, they struggled to maintain precise context-sequence mappings under conditions of contextual instability.

4.3.7. Sliding window performance for the reversal learning

Behavioural data were collected across a series of trials for Monkey PL during the reversal learning phase. For each trial, performance was recorded as a binary outcome, and trials were ordered sequentially.

To visualise changes in performance across trials, a sliding window approach with a window size of 80 trials was applied. This window size was chosen based on an analysis of breakpoint stability across different window sizes: smaller windows (< 80 trials) produced volatile breakpoint estimates due to trial-by-trial noise, whereas larger windows (≥ 80 trials) produced consistent estimations. This approach generated a smoothed estimate of performance for each trial, referred to as the 'sliding performance'.

A segmented (piecewise) regression analysis was performed to determine the trial at which performance stabilised. First, a linear regression model (sliding performance ~ trial number) was fitted, and then a segmented regression was applied to identify a breakpoint where the rate of performance change markedly slowed. Trial number served as the independent variable, and sliding performance as the dependent variable. Model fit was evaluated using residual standard error and adjusted R^2 values.

The segmented regression analysis revealed a significant breakpoint in the learning curve at Trial 1585 (SE = 17, Figure 4.10). Prior to the breakpoint, performance improved steadily at a rate of 0.045% per trial (p < .001). After the breakpoint,

performance plateaued, indicating the transition from active learning to stable task performance. The segmented model explained 70.4 % of the variance in performance (Adjusted R^2 = 0.7038), reflecting an excellent fit. These results suggest that approximately 1585 trials were required for Monkey PL to reach stable performance on the reversal learning task.

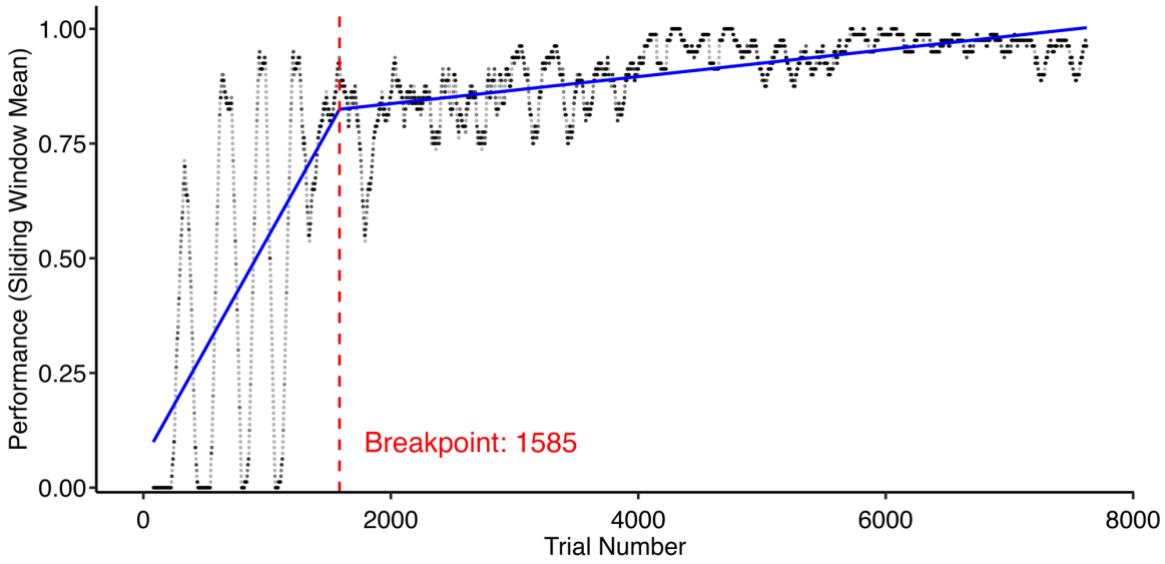

Figure 4.10. Sliding window performance with segmented regression analysis.

Performance across trials for reversal learning in Monkey PL is shown as grey points each representing the mean performance within a sliding window of 80 trials. The blue line represents the fitted values from a segmented regression model, highlighting the transition from an initial learning phase to a stable performance phase. The red dashed line indicates the estimated breakpoint at Trial 1585 (SE = 17), where the rate of performance improvement markedly slowed. The breakpoint reflects the estimated trial at which learning stabilised.

4.4. Discussion

This chapter investigated the influence of context on sequential object selection in macaques, focusing on error patterns and performance differences across stable and unstable contexts. The results illuminate the interaction between context and sequence learning, emphasising the stabilising effect of consistent environments and the cognitive demands posed by dynamic contextual shifts.

The task required macaques to select objects in a specific sequence using touchscreens attached to their home-unit. During the stable context phase, the contextual associations between objects and their environment remained consistent across trials. This consistency enabled the animals to form and rely on fixed context-object mappings to guide their choices, underscoring the role of stable environmental cues in facilitating associative learning. Notably, the majority of errors occurred when selecting the second object in the sequence. Error patterns indicated a tendency toward perseveration, with animals frequently repeating their initial object choice for the second element of the sequence rather than completing the sequence as required.

Several factors may account for this behaviour. First, the salience of the association between the first object and the relevant context may have overshadowed the subsequent element of the sequence. Since the first object was always required, the frequent reinforcement of this step might have disproportionately strengthened its association with the context. Secondly, the macaques received rewards after every object selection, which may have further reinforced memory of the initial object, making the transition to the subsequent object less cognitively salient.

The reversal phase posed a unique challenge that was not conducted with the rats and marmosets in previous chapters. In this phase, macaques were required to relearn object sequences with reversed object-context associations. While the sequences and the spatial stability of the context in the animals' home-unit remained unchanged, the context to which the sequence was associated was switched. Results revealed high error rates when selecting the first object, compared to the second object or perseverative errors (in repeating the first object for the second choice), indicating it was initial recollection that interfered with performance.

This interference effect likely occurred because the macaques, upon encountering the new context, recalled the previous context-sequence association rather than adopting

the newly associated sequence. Despite this difficulty, analysis of error types showed no significant differences between the two contexts, suggesting that once the animals correctly identified the first object, they were more likely to maintain and complete the sequence successfully.

The unstable context phase introduced dynamic contextual changes, requiring the macaques to adapt to shifting environmental cues. This phase imposed greater cognitive flexibility and heightened demands for working memory and inhibitory control. Both first and second object selection were affected as a result, with second object errors occurring significantly more often when the context shifted mid-trial. These findings align with theories of working memory and cognitive control, which suggest that updating task-relevant information in response to environmental changes is inherently challenging (Friedman & Robbins, 2022).

Error patterns for the unstable context phase revealed a bias toward order and context errors, both of which occurred with similar frequency when a contextual switch was involved. The similarity in error frequencies suggests that the macaques did not rely on a single distinguishing component (order or context) to make their choices but instead struggled to integrate multiple factors simultaneously.

Order errors occurred when the object chosen was correct for the current context but was selected out of sequence. For example, in a trial shifting from Blue to Yellow context, choosing object C (in the Yellow context) instead of object D (the correct second object in the sequence) constituted an order error. This error suggests the animals perceived the contextual shift as potentially marking the start of a new trial, leading them to reset their response sequence. This interpretation is further supported by the fact that the start of the trial was signalled, but not the end, which may have led the macaques to perceive the context switch as the trial's conclusion and initiate a new sequence.

Context errors, in contrast, involved selecting objects that adhered to the sequence order but belonged to the wrong context. Again, using the example above, choosing object B (the second object in the Blue sequence) instead of object D in a trial shifting from Blue to Yellow context would reflect a context error. This type of error suggests that the macaques did not perceive the trial as having changed context or were unable to inhibit preplanned responses.

Order errors may be attributed to the animals' interpretation of the contextual shift as the onset of a new trial rather than a continuation of the same trial with a rule change. This misinterpretation likely stemmed from the animals' prior experience, where each trial was delineated by a white spot cue and followed a single, consistent context. The transition from one colour to another in the unstable phase may have been perceived as signalling a new trial, leading to the initiation of the sequence from the beginning.

Conversely, context errors suggest a failure of inhibitory control, with animals proceeding with preplanned responses despite the contextual change. Although objects were randomly arranged at the start of each trial, their arrangement remained unchanged mid-trial, potentially allowing the animals to plan their responses in advance. This planning, coupled with a lack of flexibility to adjust to new rules, may explain the persistence of context errors.

It is worth considering whether alternative methods could have been employed to ensure that the macaques noticed the background colour change. For instance, incorporating a more distinct or overt cue to signal the context switch (e.g., an auditory tone or a brief visual flash) could have made the transition more noticeable. Additionally, providing a clearer indication that the trial had not ended, such as a brief cue or feedback following the contextual shift, might have helped the macaques better track the continuation of the trial despite the change. However, these adjustments could also introduce new variables that might impact the animals' performance in other ways, such as increasing cognitive load or distracting attention from the core task. Thus, while alternative cues could potentially improve context recognition, they would need to be carefully balanced with the task demands to avoid confounding effects.

In conclusion, this chapter highlights the role of context stability in facilitating sequential object selection and learning in macaques. Stable contexts allowed the formation of robust context-object associations, reducing errors and enabling consistent performance. In contrast, unstable contexts introduced significant cognitive demands, requiring flexibility to adapt to the dynamic contextual shifts. The observed error patterns, including perseverative errors, order errors, and context errors, underscore the challenges posed by changing environmental cues and the reliance on both inhibitory control and sequence updating for successful task completion. Together, these findings provide valuable insights into the interplay between context,

memory, and cognitive flexibility during context-dependent sequence learning. They also provide a robust foundation for utilising transcranial ultrasound stimulation to investigate the neurobiological mechanisms underlying this type of memory.

Chapter 5: Enhancing Context-Guided Memory with Transcranial Ultrasound Stimulation in Rhesus Macaques

5.1. Introduction

Episodic memory – the capacity to recall specific past events within a contextual framework – is a cornerstone of human cognition, enabling navigation of complex social and environmental challenges. Understanding episodic processes has far-reaching implications, not only for advancing cognitive neuroscience but also for addressing memory-related disorders, including Alzheimer's disease and post-traumatic stress disorder. Insights into the underlying neural circuits can inform the development of targeted interventions aimed at restoring or enhancing memory function.

As it currently stands, understanding of the neurobiological underpinnings of episodic memory, or episodic-like memory in nonhuman primates, remains limited and largely extrapolated from rodent studies. While rodent models have elucidated the role of prefrontal-hippocampal connectivity in context-guided tasks, translating these findings to primates is challenging due to differences in animal size and functional organisation of the brain. Namely, primates are much larger and so creating a free-moving task that accommodates shifting contexts is difficult and costly. In addition, the techniques used to investigate neural function such as optogenetics and electrophysiology require the animal to be restrained, further limiting the translatability between the two.

The prefrontal-hippocampal circuit is critical for encoding, consolidating, and retrieving episodic memories, with specific regions contributing differentially to various aspects of memory processing. Along the hippocampal axis, the anterior hippocampus (aHPC) is associated with encoding larger spatial environments and overarching contextual frameworks (Ryan et al., 2010; Nadel et al., 2013), supported by direct projections to the medial prefrontal cortex (mPFC). In contrast, the posterior hippocampus (pHPC) integrates finer environmental details, such as spatial cues, and supports object-place associations and context-dependent learning. This region operates independently of direct medial prefrontal input but communicates indirectly via structures such as the nucleus reuniens and entorhinal cortex (Komorowski et al., 2009; Burwell & Amaral, 1998).

The mPFC, on the other hand, is required for executive control, schema integration, and resolving memory interference through indirect connections to the hippocampus. The dynamic interplay between these regions (thought to be mediated by theta oscillatory synchrony) facilitates context-dependent memory selection. During encoding, hippocampal theta precedes medial prefrontal activity, reflecting the transfer of contextual information. During retrieval, this flow reverses, with medial prefrontal theta guiding hippocampal processes to support accurate memory selection (Benchenane et al., 2010; Place et al., 2016). The nucleus reuniens appears to synchronise these oscillations, enabling bidirectional communication between these regions (Dolleman-van der Weel et al., 2019).

Recent advancements in non-invasive neuromodulation highlight transcranial ultrasound stimulation (TUS) as a transformative tool for targeting deep brain structures, such as the hippocampus and prefrontal cortex. Unlike transcranial magnetic stimulation or transcranial direct current stimulation, TUS offers high spatial selectivity and deep brain penetration, enabling precise modulation of subcortical structures typically accessible only though invasive methods (Bystritsky et al., 2011). According to the Neural Intramembrane Excitation model, TUS modulates neuronal activity by influencing mechanosensitive ion channels through localised mechanical vibrations propagated through the brain in the form of ultrasound waves (Plaksin et al., 2014).

The non-invasive nature of TUS is especially advantageous for animal research, aligning with ethical principles of refinement and reduction in animal use (Hubrecht & Carter, 2019). Historically, investigating specific brain regions often required lesioning or irreversible modification in animal models, with multiple individuals being required to model multiple different lesions. In contrast, TUS permits repeated interventions with reversible effects that avoid long-term damage (Gaur et al., 2020), allowing for multiple regions to be assessed within-subjects. This capability makes TUS an ideal tool for bridging the gap between rodent and primate research, enabling causal investigations of episodic (or episodic-like) memory processes in context-dependent tasks.

This study aims to test the hypothesis that TUS can causally modulate distinct aspects of context-dependent memory by targeting specific regions of the prefrontal-

hippocampal circuit. Specifically, three key regions will be stimulated independently using a rigorous and counterbalanced design: the aHPC, pHPC and mPFC. These regions are hypothesised to contribute differentially to the encoding, retrieval, and resolution of context-dependent memory.

The aHPC is hypothesised to be critical in encoding and recalling generalised representations of an events. Evidence from human functional imaging studies indicates that human anterior hippocampal activation increases during the retrieval of general contextual information (Liang et al., 2013). Thus, TUS applied to the aHPC is expected to enhance the encoding and recall of broader contextual information.

The pHPC is believed to support fine-grained spatial and context-specific object-place encoding (Komorowski et al., 2009). TUS modulation of pHPC activity is anticipated to improve performance requiring precise object-location disambiguation. Disruptions may occur, however, if task demands alter spatial consistency between events, reflecting the pHPC's sensitivity to precise, stable object-place associations.

The mPFC is hypothesised to guide memory retrieval by leveraging relevant contextual cues to resolve conflicting information. Previous findings in rodent studies demonstrate that mPFC inactivation impairs task switching, particularly when faced with competing spatial contexts (Rich & Shapiro, 2007). Therefore, TUS stimulation of the mPFC is predicted to improve task flexibility and reduce errors, especially during mid-trial contextual shifts.

By combining behavioural data with neuromodulation, this work seeks to validate and extend rodent findings in nonhuman primates, investigating the neural mechanisms underlying context-dependent sequence learning. This chapter explores the potential of TUS to elucidate the contributions of the prefrontal-hippocampal network to context-dependent sequence learning in nonhuman primates. By leveraging TUS's non-invasive capabilities, it aims to bridge the gap between rodent and primate studies, providing novel insights into episodic memory mechanisms and establishing a foundation for translational applications in memory-related disorders.

5.2. Methods

5.2.1. Subjects

Two rhesus macaques (*Macaca mulatta*) participated in the experiment: one male (PL, 16 years old, weighing 14 — 15 kg) and one female (MC, 9 years old, weighing 6 — 7 kg). Both macaques were pair-housed with other males (PL with a male companion, and MC with a different male). The primate colony at Newcastle University comprises approximately 40 macaques housed in compatible social pairs or triplets. Each pair was housed in a home-unit (L: 2.4 m, W: 1.4 m, H: 2.3 m) with an adjoining area (corridor) between units (Figure 5.1A).

The colony was maintained on a 12-hour light-dark cycle (lights on from 07:00 to 19:00) with controlled environmental conditions: a stable temperature of 16 – 25 °C and relative humidity of 40 – 70 %. All animals were under a fluid control protocol approved by the Home Office, tailored to each individual's needs. This protocol ensured sufficient hydration (at least 20 ml/kg daily) while motivating participation in tasks. Testing sessions were conducted during the light phase, consistently scheduled from 09:00 to 13:00.

All procedures adhered to the guidelines of the UK Animals (Scientific Procedures) Act of 1986, were approved by Newcastle University's Animal Welfare and Ethical Review Body and complied with the European Directive on the protection of animals used for scientific purposes (2010/63/EU). Reporting of this study follows the recommendations in the ARRIVE guidelines.

5.2.2. Apparatus

Behavioural testing involved two touchscreens mounted on two adjacent home-units, separated by a central corridor (Figure 5.1A). A juice spout, centrally positioned below each screen, dispensed a fluid reward (Ribena Blackcurrant Juice, Suntory, Bristol, UK) upon correctly completing a response.

5.2.3. Materials

The on-screen stimuli (Figure 5.1B) were photographs of the real objects used in the rodent experiment (see Chapter 2). Each image measured 200 x 200 pixels, with objects presented on a neutral grey background, distinct in shape and colour to ensure clear visual differentiation. The experiment was conducted using PsychToolbox

Version-3 (Brainard, 1997; Pelli, 1997; Kleiner et al., 2007) within MATLAB (version 2017a/2018a), running on a Windows 10 Enterprise machine connected to each touchscreen.

5.2.4. Design

Prior to each testing session, the participating macaque was temporarily separated from its companion. Touchscreens were securely attached to the home-units as described earlier, and both screens were connected using a with a coaxial cable (BNC, Bayonet Neill-Concelman) attached to LabJack (U3, https://labjack.com) devices on each machine. This configuration ensured both touchscreens operated simultaneously on the same task.

An example trial is illustrated in Figure 5.1C. To initiate a trial, the macaque first touched a white circle displayed at the centre of the screen. Following a 1-second inter-stimulus interval (ISI), the stimuli appeared on the screen, prompting the animal to make a choice. The time taken to make this initial touch was recorded as 'Response 1' reaction time (RT). If the choice was correct, the macaques received a predefined volume (~1.5 ml) of juice as a reward. An incorrect choice resulted in the trial being aborted, and a white circle reappeared to start the next trial.

Upon a correct first response, the macaque could make a second choice. If correct, a second juice reward (~ 1.5 ml) was dispensed, and the time taken to make this response was recorded as 'Response 2' RT. After a 1-second inter-trial interval, the next trial became available. Incorrect second responses resulted in trial termination without a reward, and the white circle reappeared to initiate the next trial.

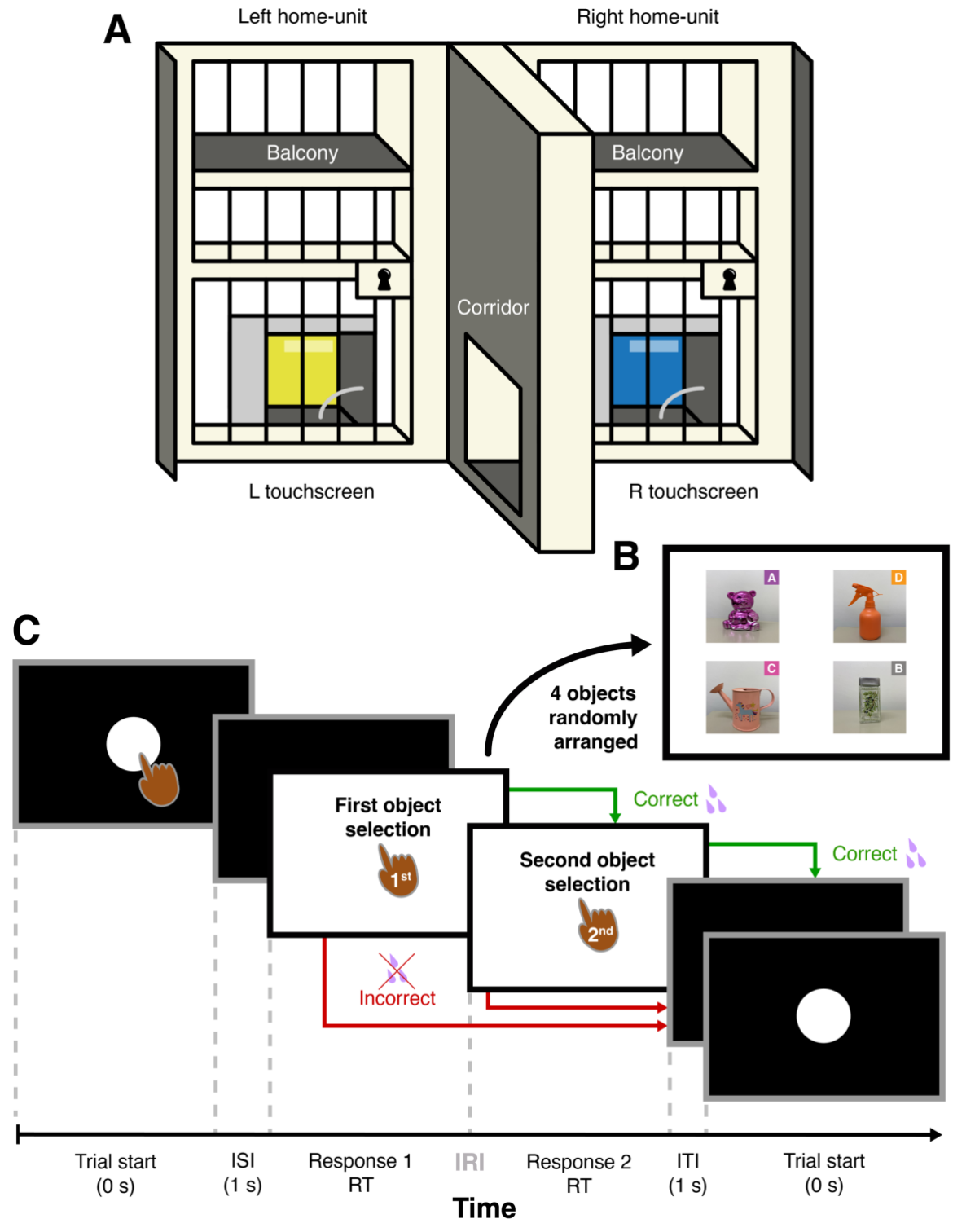

Figure 5.1. Schematic representation of the home-unit setup and trial sequence.

A) *The touchscreen setup consisted of two home-units connected by a central corridor. Each home-unit contained a touchscreen within reach of the animal.* ***B)*** *Depicts the time-course and sequence of events in a typical trial and* ***C)*** *Displays the four stimuli images used. Images were randomly organised each trial. Abbreviations: Inter-stimulus Interval (ISI); Reaction Time (RT); Inter-trial Interval (ITI).*

5.2.5. Ultrasound Stimulation Parameters

A single-element ultrasound transducer (H115-MR, Sonic Concepts, Bothell, WA, USA) was used for this study. Operating at a centre frequency of 250 kHz, the transducer featured a curvature radius of 63.2 mm and an aperture diameter of 64 mm (Figure 5.2A, Table 5.1). The focal depth was fixed at 51.74 mm, with a focal beam size of 39.5 x 6.04 mm (length x width; Figure 5.2B). To achieve a desired focal depth of 21.74 mm from the skull surface, a 30-mm coupling cone was attached to the transducer housing.

5.2.5.1. Drive system components

The system included a KeySight 33500B Trueform signal generator (KeySight, Santa Rosa, CA, USA) to provide the input signal required for transducer excitation. A TBS 1032B oscilloscope (Tektronix, Beaverton, OR, USA) was used for real-time signal visualisation of the signal during sonication. Signal amplification was achieved using a 75-W Model 7500 amplifier (Krohn-Hite, Brockton, MA, USA). To optimise impedance matching between the amplifier and the transducer, an electrical impedance matching network (Sonic Concepts) was included.

5.2.5.2. Drive system settings

Sonication parameters were generated using a Windows 10 Enterprise computer and relayed to the signal generator (KeySight 33500B) via a LabJack (U3 Model). A 30-mm Perspex coupling cone filled with degassed water and sealed with a latex membrane was attached to the transducer housing to achieve the required focal length.

5.2.5.3. Free field acoustic parameters

Free-field acoustic measurements were performed in a water bath using a 1-mm needle hydrophone (NH100, Precision Acoustics, Higher Bockhampton, DO, UK) with a measurement uncertainty of 9 %. At the focal depth of 51.74 mm, the spatial peak pressure amplitude was approximately 580 kPa, with a spatial-peak pulse-average intensity (I_{sppa}) of 11.5 W / cm ^{2} and a spatial-peak temporal-average intensity (I_{spta}) of 3.45 W / cm ^{2}.

Table 5.1. Transducer and Pulse Parameters.

Category	Parameter	Value
Transducer	Manufacturer (Model Number)	Sonic Concepts (H115-MR)
	Centre Frequency	250 kHz
	Radius of Curvature	63.2 mm
	Aperture Diameter	64 mm
	Number of Elements	1
Matching Network	Manufacturer	Sonic Concepts (Electrical impedance matching network)
Pulse Timing	Pulse duration	0.03 sec
	Ramp Duration	0 sec
	Ramp Shape	Rectangular
	Repetition Interval / Frequency	0.1 sec / 250 kHz
	Pulse Train Duration	40 sec
	Pulse Train Ramp Shape	Rectangular

5.2.5.4. Pulse timing parameters

Active ultrasound stimulation involved 30-millisecond pulses (pulse duration) delivered every 100 milliseconds (pulse repetition interval), corresponding to a 30 % duty cycle. The total pulse train duration lasted 40 seconds (Figure 5.2C, Table 5.1). The peak-to-peak voltage remained constant throughout the stimulation.

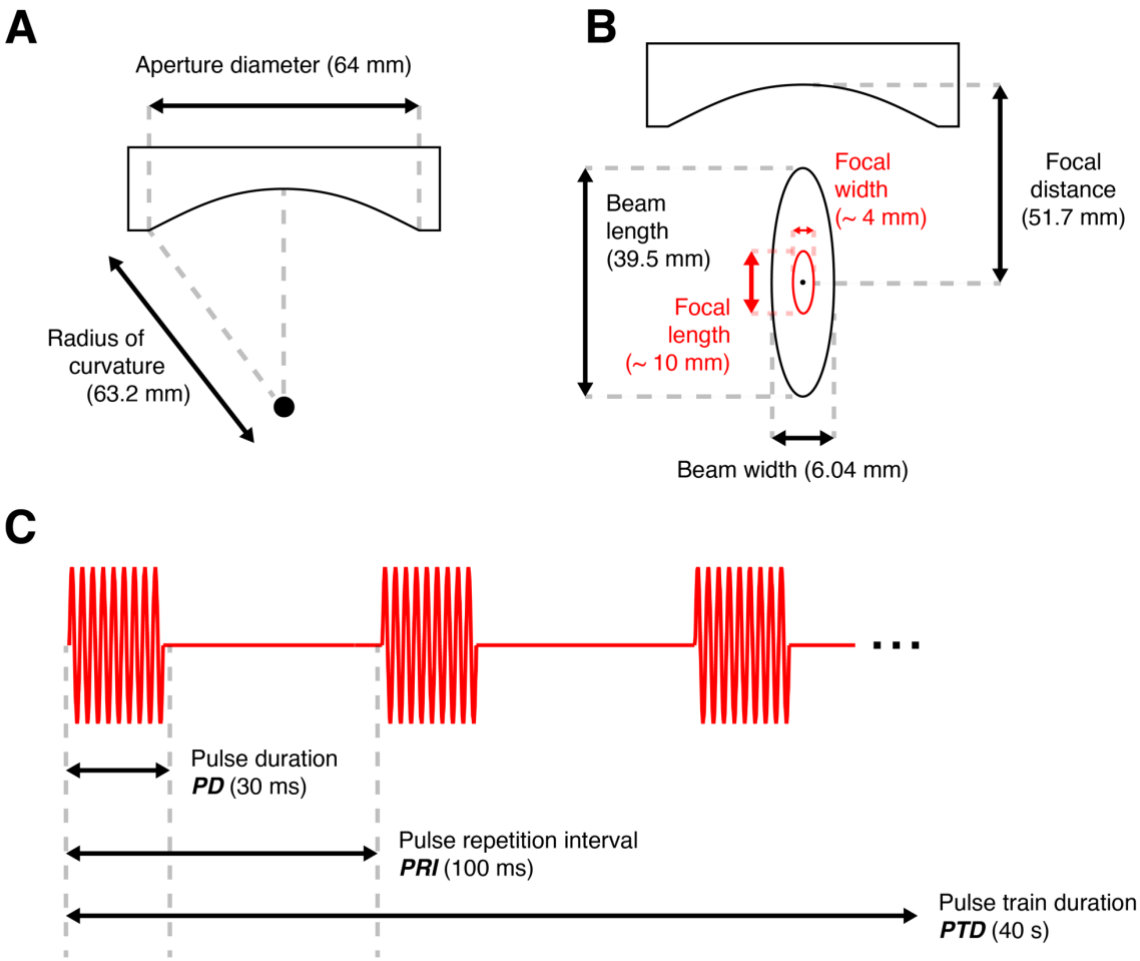

Figure 5.2. Transducer dimensions and waveform parameters.

A) *Diameter of the transducer and* ***B)*** *Parameters of the focal distance, beam length and beam width.* ***C)*** *Schematic of the ultrasound pressure waveform.*

5.2.5.5. Application of sonication

Prior to sonication, the transducer was filled with room-temperature water and sealed with a latex membrane and O-ring to eliminate air bubbles. The transducer was then calibrated to a frameless stereotaxic neuronavigation system (BrainSight Vet, Rogue Research, Montreal, CAN) using fiducial markers aligned parallel to the transducer housing. Six fiducial markers were attached the animal's head-post, along with a subject tracker, allowing for calibration of the animal's head position and orientation within BrainSight. Earplugs were used throughout the sonication to protect the animal and reduce the impact of noise-related confounds.

Conductive ultrasound gel (Cutimed, Hull, Yorks, UK) was applied generously to the latex membrane. The transducer was positioned perpendicular to the target area on the scalp as guided by BrainSight and held in place for the full 40-second sonication period. Sonication was performed bilaterally in each session, with the order of hemispheres (left or right) alternated between sessions.

5.2.6. Target Brain Areas

Each macaque underwent an awake T1-weighted structural magnetic resonance imaging (MRI) scan to create a template for targeting three specific brain regions: the medial prefrontal cortex, anterior hippocampus and posterior hippocampus. Scans were acquired using a 4.7 T Bruker Vertical scanner. Six fiducial markers were affixed to the animal's headpost to enable precise co-registration with the BrainSight neuronavigation system.

The acquired MRI scans were uploaded to the BrainSight platform where the D99 version 2.0 atlas (Saleem et al., 2021) was used as a reference to identify and map the fiducial markers and the target brain regions for sonication.

Figure 5.3 illustrates the three targeted brain regions with the focal ellipsoid of the ultrasound beam overlaid, measuring 10 mm in length, 4 mm in width, and 4 mm in height. The specific coordinates of the three targeted regions are as follows: medial prefrontal cortex (x: 126; y: 244; z: 110, Figure 5.3A), anterior hippocampus (x: 90; y:177; z: 66, Figure 5.3B), and posterior hippocampus (x: 85; y: 121; z: 115, Figure 5.3C). This mapping allowed for precise delivery of ultrasound stimulation to the intended brain areas.

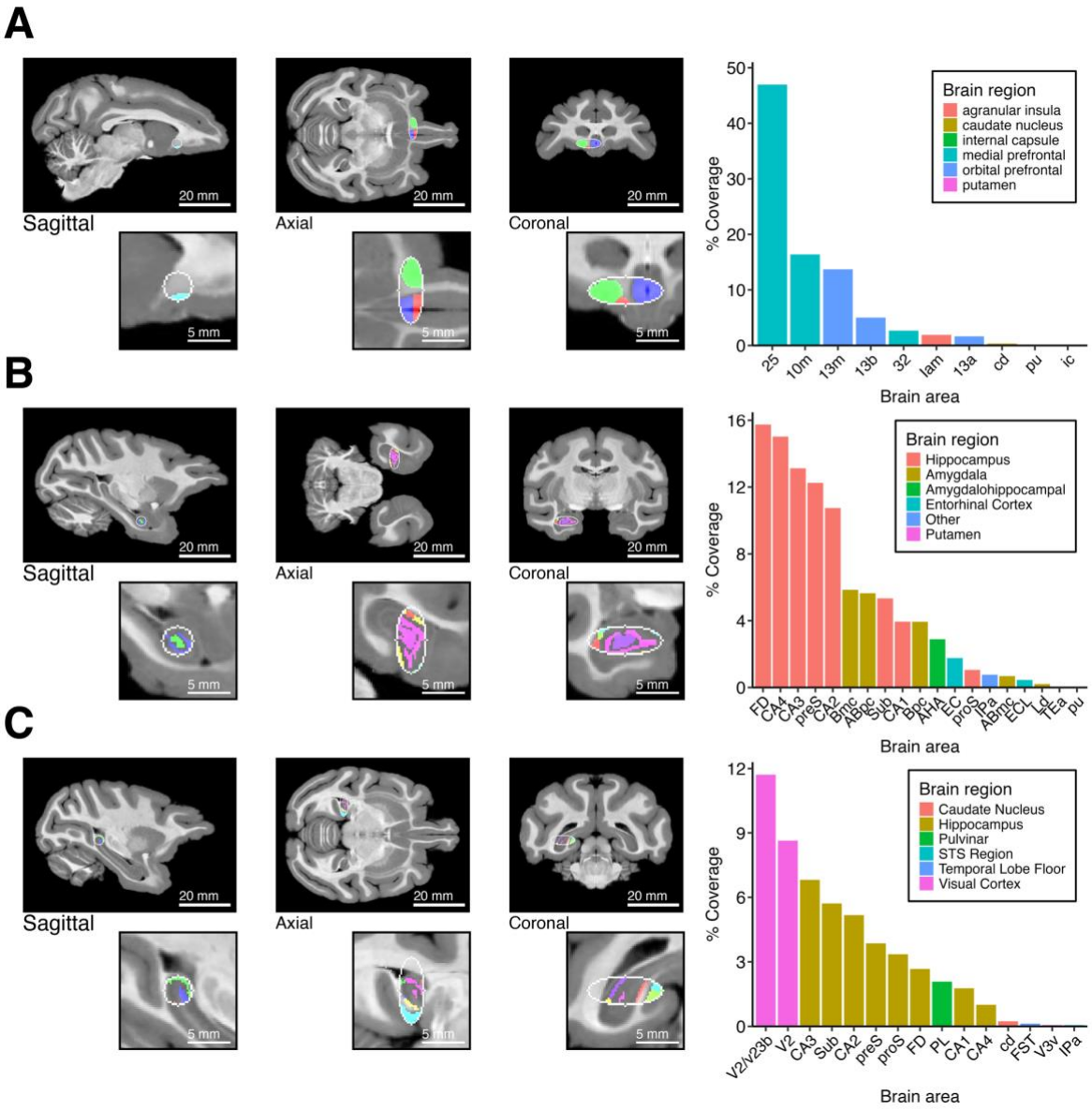

Figure 5.3. Brain regions targeted by ultrasound stimulation and their corresponding coverage in the focal ellipsoid beam.

The panels illustrate the ultrasound focal beam targeting three brain regions in macaques: ***A)*** *medial prefrontal cortex,* ***B)*** *anterior hippocampus, and* ***C)*** *posterior hippocampus. Images in sagittal, axial, and coronal planes show the spatial distribution of the ultrasound stimulation (white ellipsoid) in each region. Below each set of images, magnified views detail the focal area. The bar graphs on the right indicate the percentage of coverage across specific brain regions within the focal ellipsoid. Different colours correspond to key brain structures affected, according to the D99 2.0 atlas. The percentage distribution quantifies the extent of inclusion for each brain region within the focal area. Please note, colours are not consistent between scans or bar graphs. The focal ellipsoid beam measured L:10, W: 4, H: 4 mm.*

5.2.7. Behavioural Testing

The detailed training and testing schedule for learning the different phases of the experiment is outlined in Chapter 4 (Section 4.2) but will be summarised here.

Habituation to the touchscreen involved two sessions per animal, allowing them to associate touching objects onscreen with receiving a fluid reward (Section 4.2.5). During this stage, objects were presented individually without incorrect trials, enabling familiarisation with the objects and background colours. Following habituation, animals progressed through increasingly complex training stages (Section 4.2.6).

To minimise distractions, each animal used a single touchscreen per session, with touchscreen consistently assigned to specific sides of the home-unit. Trials were counterbalanced across sessions to prevent biases towards a particular touchscreen, object set, or background colour.

In the initial training phase, pairs of objects were presented simultaneously in fixed spatial locations, teaching the animals the relevance of object identity and sequence order within each context (Section 4.2.6.1). In subsequent phases, object locations (Section 4.2.6.2), encouraging reliance on object identity over spatial position. Additional reinforcement was provided to one monkey (monkey MC) to strengthen the reliance on sequence order over spatial location.

Later training phases introduced incongruent objects, requiring animals to disregard certain objects based on the background colour (Section 4.2.6.3). The final training phase emphasised that spatial location was irrelevant for both congruent and incongruent objects (Section 4.2.6.4).

During testing (Section 4.2.7), two touchscreens were mounted on either side of the home-unit, each presenting a distinct context defined by background colour. In the Stable context phase (Figure 5.4A), Yellow context trials were always presented on left screen and Blue trials on the right. Animals learned that the correct object sequence was object A followed by object B in the Yellow context and object C then object D in the Blue context. To balance experience, the active touchscreen switched after every five trials, prompting the animals to move between screens to continue the experiment.

Subsequent testing phases evaluated adaptability. In the Reversal learning phase (Figure 5.4B), contexts remained spatially stable, but the correct object sequences were switched: Yellow context trials now required selecting object C then D, and Blue context trials required object A followed by object B.

In the final testing phase (the Unstable context phase, Figure 5.4C), context assignment became random across touchscreens. Trials introduced mid-sequence context switches; for example, a trial beginning with a Yellow background (requiring object C) might switch to Blue mid-sequence, necessitating selection of object B. Successful performance required dynamic updating of choices based on the currently active context.

Prior to each testing session, stimulation was administered in the laboratory, with animals briefly transported from their home unit and returned immediately following stimulation. The time from stimulation completion to task onset was approximately 20 minutes. The stimulation procedure was bilateral, lasting 40 seconds per hemisphere (Section 5.2.5.5), and conducted while animals were awake, as it was non-invasive and painless. Evidence from similar studies using the same post-stimulation ("offline") approach and neuroimaging methods suggests that TUS can induce transient brain changes lasting from several minutes to several hours (Atkinson-Clement et al., 2025). In primates, stimulation of cortical and subcortical regions has been shown to alter functional connectivity for over an hour (Folloni et al., 2019; Verhagen et al., 2019), and changes in task performance have been observed more than 30 minutes post-stimulation following stimulation of the anterior cingulate cortex (Fouragnan et al., 2019).

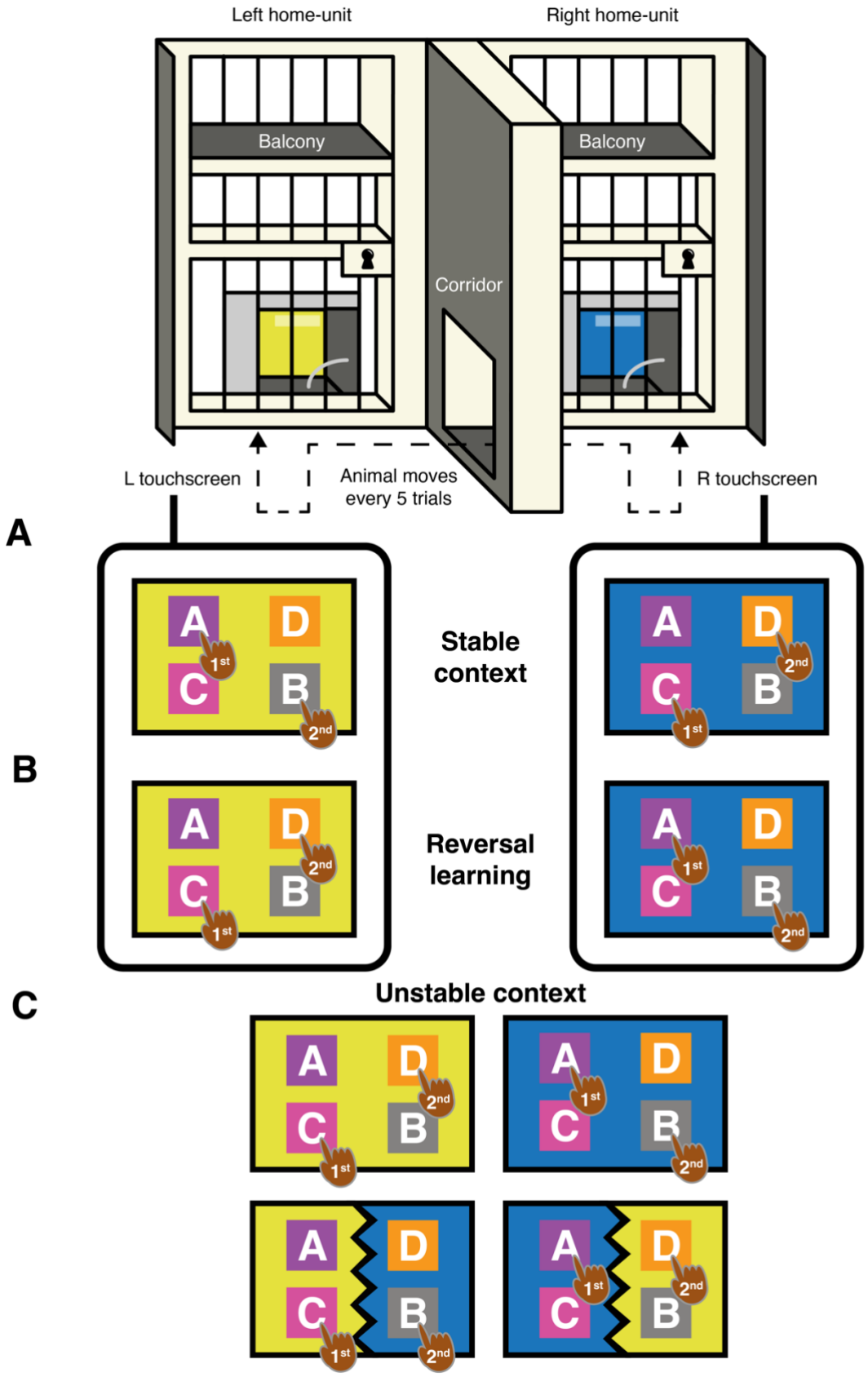

Figure 5.4. Testing procedure phases illustrating context and task variations.

A) *Stable context phase: In the first testing phase, the left touchscreen consistently displayed Yellow context trials (correct sequence: object A followed by object B), while the right touchscreen displayed only Blue context trials (correct sequence: object C followed by object D).* ***B)*** *Reversal learning phase: The second phase involved a reversal of the correct object sequences within each context. The Yellow context (still on the left touchscreen) now required the selection of object C followed by object D, and the Blue context (still on the right touchscreen) required the selection of object A followed by object B.* ***C)*** *Unstable context phase: In the final phase, the context association with the screens became unpredictable with any of four trial types appearing on either screen. Trials could either belong to familiar contexts (Yellow or Blue trials as seen in the Reversal phase) or involve a mid-trial contextual switch (Yellow-to-Blue or Blue-to-Yellow trials).*

5.3. Results

5.3.1. Testing sequence comprehension for stable contexts

In the initial testing phase, the two touchscreens were attached to adjacent sides of the home-unit separated by the central corridor (Figure 5.4). Each screen displayed one specific context: Yellow context trials were presented on the left screen, while Blue context trials appeared on the right (Figure 5.4A). To encourage animals to engage with both screens, and ensure an equal number of trials per context, the active touchscreen alternated every five trials, irrespective of performance. This setup likely engaged the prefrontal-hippocampal network by requiring the animals to integrate the two sequences within distinct contexts. Contextual cues could be egocentric (e.g., the macaques' physical location by the touchscreen) or allocentric (e.g., the background colour), paralleling how contextual memory operates in humans.

For monkey PL, the experiment followed a block design, with repeated stimulation of one brain region across several sessions, within which, sham sessions were counterbalanced. This approach required grouping sessions by blocks during analysis to control for potential learning effects. Conversely, for monkey MC, all brain areas were counterbalanced across the entire experiment with sham sessions, removing the need for block grouping.

Monkey PL's performance was analysed using a binomial generalised linear mixed model with brain region and session block as fixed predictors and session block as a random effect to account for variability across blocks. The analysis revealed significant effects of brain region stimulation on task performance (Figure 5.5A-B). Specifically, stimulation of the anterior hippocampus (mean: 94.1 % ± SE: 1.59, Figure 5.5A) significantly enhanced performance compared to sham trials (reference condition; mean: 91.7 % ± SE: 2.09, $ß$ = 0.366 ± 0.113, z = 3.25, p = 0.001, Figure 5.5B). Conversely, stimulation of the posterior hippocampus significantly impaired performance (mean: 84.9 % ± SE: 3.95, $ß$ = - 0.683 ± 0.193, z = - 3.53, p < 0.001, Figure 5.5B). These findings indicate that anterior hippocampus stimulation is important for recalling object sequences within stable contextual environments, whereas the posterior hippocampus may hinder this process. In addition, stimulation of the medial prefrontal cortex (mean: 94.9 % ± SE: 1.99, Figure 5.5A) did not significantly influence performance ($ß$ = 0.509 ± 0.340, z = 1.50, p = 0.134, Figure

5.5B). This lack of medial prefrontal involvement could be attributed to the stability and distinctiveness of the two contexts, both of which were reinforced by fixed spatial boundaries. Such stability likely enabled the hippocampus to independently maintain sequence comprehension.

The analysis also accounted for the influence of experimental block design, showing that Monkey PL's performance varied significantly across different blocks (ß = 1.04 ± 0.334, z = 3.11, p = 0.002, Figure 5.5B), with performance steadily improving over the course of testing, regardless of stimulation. The observed performance improvement across blocks suggests that repeated exposure and familiarity with the task facilitated learning, potentially masking subtler contributions of the medial prefrontal cortex.

For monkey MC, brain regions were counterbalanced throughout the experiment, allowing for analysis using a binomial logistic regression model with brain region as a fixed predictor and . Similar to monkey PL, stimulation of the anterior hippocampus significantly improved performance (mean: 92.7 % ± SE: 1.11, Figure 5.5C) compared to sham trials (reference condition; mean: 89.1 % ± SE: 1.14, ß = 0.434 ± 0.201, z = 2.16, p = 0.031, Figure 5.5D). The consistency of these findings across both monkeys reinforces the importance of the anterior hippocampus in stable context comprehension, possibly by encoding sequences into broader contextual frameworks.

In further support of monkey PL's results, posterior hippocampal stimulation (mean: 85.2 % ± SE: 1.84, Figure 5.5C) showed a marginally non-significant negative effect on performance (ß = - 0.356 ± 0.187, z = - 1.90, p = 0.057, Figure 5.5D). Similarly, stimulation of the medial prefrontal cortex did not significantly affect performance (mean: 83.8 % ± SE: 3.47, ß = - 0.465 ± 0.281, z = - 1.65, p = 0.098, Figure 5.5D). These results provide additional evidence that stable contextual task performance depends on the integrative capacity of the anterior hippocampus, rather than the detailed spatial processing of the posterior hippocampus or ability to resolve ambiguity associated with the medial prefrontal cortex.

5.3.2. Testing sequence comprehension when relearning sequence order

The subsequent phase, conducted exclusively with monkey PL, involved relearning the context-sequence associations. The pair of objects previously relevant to the Yellow context (objects A and B) was now reassigned to the Blue context, and vice

versa (Figure 5.4B). Sessions were appropriately counterbalanced with sham sessions, eliminating the need for block grouping in the analysis.

A binomial logistic regression with brain region as a fixed predictor revealed that stimulation of the anterior hippocampus (mean: 89.8 % ± SE: 0.75, Figure 5.5E, *ß* = 0.889 ± 0.094, *z* = 9.42, Figure 5.5F) and the medial prefrontal cortex (mean: 85.0 % ± SE: 0.85, Figure 5.5E, *ß* = 0.451 ± 0.082, *z* = 5.53, Figure 5.5F) significantly enhanced performance compared to sham trials (reference condition; mean: 78.3 % ± SE: 0.80, $p < .001$, Figure 5.5E). Together, these findings highlight the involvement of the anterior hippocampus in maintaining an overall context identity even when previously learned associations are reversed. Meanwhile, the medial prefrontal cortex's contribution may reflect its capacity for cognitive control and adaptability, as it likely aids in re-establishing the new contextual relationships, with the previously known context.

Stimulation of the posterior hippocampus (mean: 76.8 % ± SE: 1.07, Figure 5.5E) had no effect on performance compared to sham (reference condition; *ß* = - 0.088 ± 0.076, *z* = -1.15, *p* = 0.249, Figure 5.5F), suggesting that this region may not be as crucial for the reorganisation of sequence information when context changes, instead playing a more defined role in precise spatial encoding or detailed memory during initial learning.

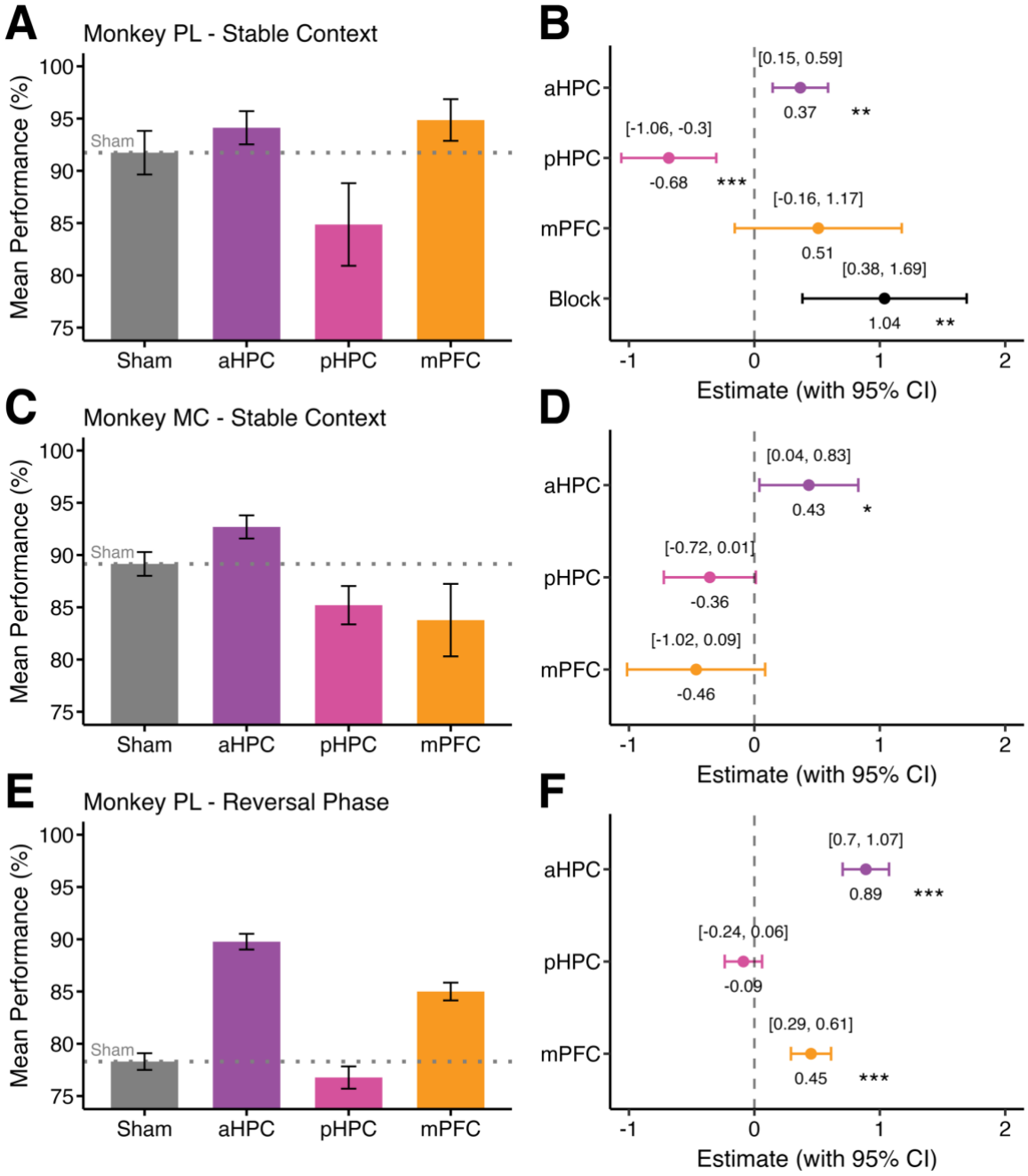

Figure 5.5. Effects of stimulation on trial performance in three brain regions compared to sham across Stable context and Reversal learning phases.

A-D) *Stable Context Phase:* ***A)*** *Mean performance (%) with standard error bars for sham, anterior hippocampus (aHPC), posterior hippocampus (pHPC), and medial prefrontal cortex (mPFC) stimulation in Monkey PL. Error bars represent the standard error of the mean (SE).* ***B)*** *Logistic regression coefficient estimates with 95 % confidence intervals (CI) for brain region effect in Monkey PL. The x-axis displays regression estimates, with the dashed line at 0 indicating no effect.* ***C)*** *Mean performance (%) with SE bars for Monkey MC across the same brain regions.* ***D)*** *Logistic regression coefficient estimates with 95 % CIs for brain region effects in*

Monkey MC. ***E-F)*** *Reversal Phase:* ***E)*** *Mean performance (%) with SE bars for Monkey PL across the same brain regions.* ***F)*** *Logistic regression coefficient estimates with 95 % CIs for brain region effects in Monkey PL. Significance is indicated by * (p < .05), ** (p < .01) or *** (p < .001).*

5.3.3. Testing sequence comprehension across unstable contexts

In the final stage of testing, the context became unpredictable within a trial as the background colour of the touchscreen could change colour after the first object was selected. The background colour (yellow or blue) could appear on either touchscreen and was not restricted to one side as it was in the first testing stage. Due to reduced motivation during testing, monkey MC was only tested on only one touchscreen per session, with the side of the home-unit to which it was attached being counterbalanced across sessions. In contrast, monkey PL continued to switch between screens every five trials.

For monkey PL, stimulation of all three brain areas significantly affected performance. Stimulation of the anterior hippocampus (mean: 74.8 % ± SE: 1.13, Figure 5.6A, *ß* = - 0.157 ± 0.075, *z* = - 2.09, *p* = 0.037, Figure 5.6B) and the posterior hippocampus stimulation (mean: 67.0 % ± SE: 1.20, Figure 5.6A, *ß* = -0.532 ± 0.071, *z* = - 7.54, *p* < .001, Figure 5.6B) both impaired performance. Conversely, medial prefrontal cortex stimulation (mean: 85.5 % ± SE: 0.86, Figure 5.6A) significantly improved performance compared to sham (reference condition; mean: 77.6 % ± SE: 0.79, Figure 5.6A, *ß* = 0.536 ± 0.083, *z* = 6.47, *p* < 0.001, Figure 5.6B).

Monkey MC also exhibited significantly improved performance following medial prefrontal cortex stimulation (mean: 80.5 % ± SE: 1.46, Figure 5.6C, *ß* = 0.399 ± 0.117, *z* = 3.41, *p* < 0.001, Figure 5.6D) compared to sham trials (reference condition; mean: 73.5 % ± SE: 1.37, Figure 5.6C). In addition, anterior hippocampus stimulation (mean: 58.3 % ± SE: 1.52, Figure 5.6C) significantly impaired performance compared to sham (reference condition; *ß* = - 0.684 ± 0.094, *z* = - 7.26, *p* < .001, Figure 5.6D). Posterior hippocampal stimulation (mean: 77.0 % ± SE: 1.31, Figure 5.6C), however, had no effect (*ß* = 0.192 ± 0.102, *z* = 1.88, *p* = 0.061, Figure 5.6D) unlike in monkey PL.

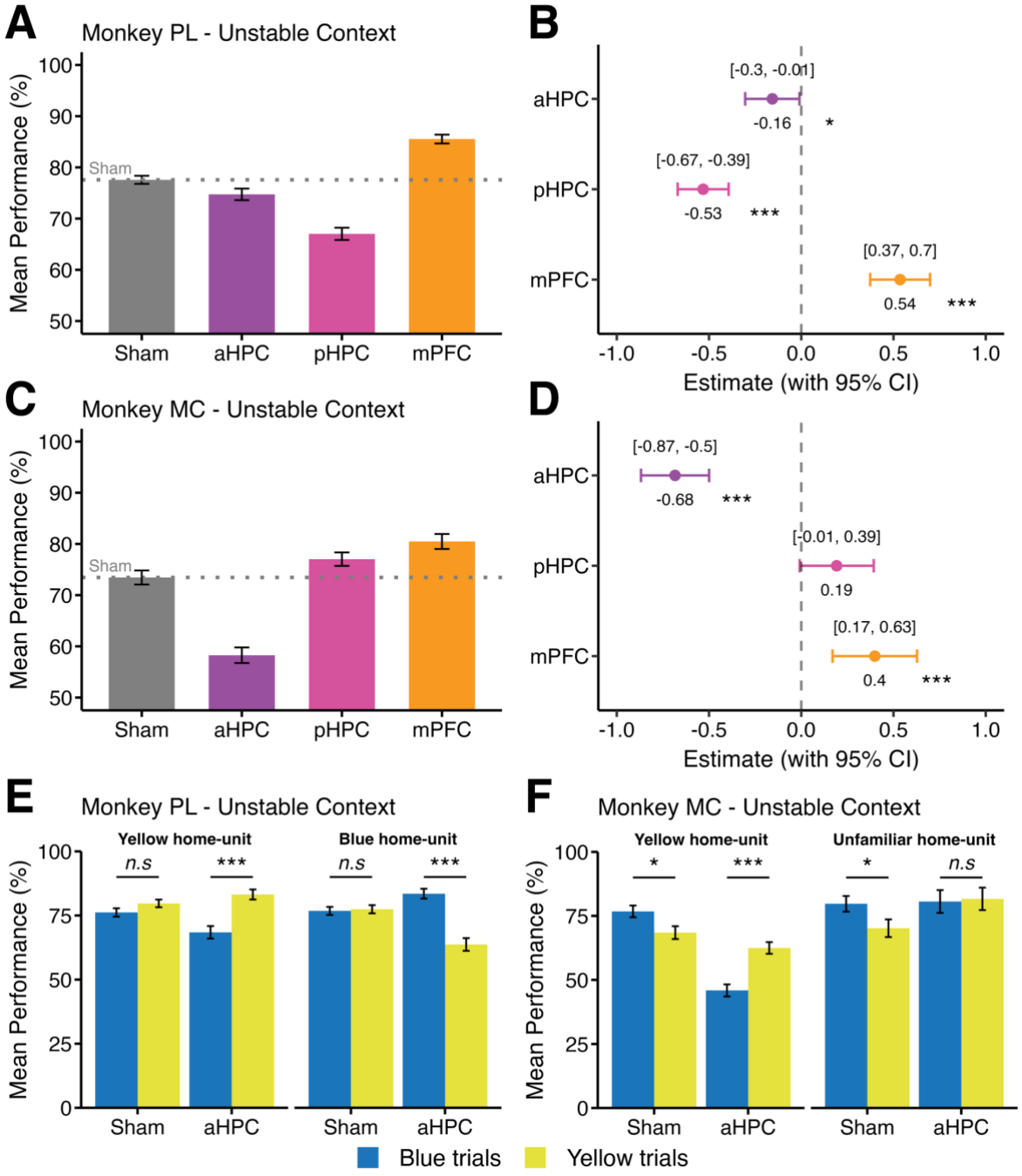

Figure 5.6. Effects of stimulation on trial performance in three brain regions compared to sham for the Unstable context phase.

A) *Mean performance (%) with standard error bars for sham, anterior hippocampus (aHPC), posterior hippocampus (pHPC), and medial prefrontal cortex (mPFC) stimulation in Monkey PL. Error bars represent the standard error of the mean (SE).* ***B)*** *Logistic regression coefficient estimates with 95 % confidence intervals (CI) for brain region effect in Monkey PL. The x-axis displays regression estimates, with the dashed line at 0 indicating no effect.* ***C)*** *Mean performance (%) with SE bars for Monkey MC across the same brain regions.* ***D)*** *Logistic regression coefficient estimates with 95 % CIs for brain region effects in Monkey MC.* ***E)*** *Mean performance*

(%) with SE bars for monkey PL for Blue and Yellow context trials tested in either the Yellow home-unit previously associated with Yellow context trials or the Blue home-unit, previously associated with Blue context trials. ***F)*** *Mean performance (%) with SE bars for monkey MC for the same trials either performed in the Yellow home-unit or a new 'unfamiliar' home-unit not directly associated with a specific context during training or testing. Significance is indicated by *** ($p < .001$), * ($p < .05$) or n.s ($p > .05$).*

5.3.4. Differences in brain stimulation for unstable context comprehension

To investigate the opposing effect of anterior and posterior hippocampal stimulation on the two primates during the Unstable context phase, additional analyses was conducted. This analysis focused on the impact of the home-unit in which the trial occurred and the context colour (background colour on the screen). Trials were performed in one of three locations: for monkey PL, who switched home-units every five trials, the trials were conducted either in the Yellow home-unit (previously associated only with Yellow context trials, on the animal's left) or the Blue home-unit (previously associated only with blue background trials, on the animal's right). Monkey MC, in contrast, performed the entire session either in the Yellow home-unit or in an adjacent 'unfamiliar' home-unit, which had not been used for training or testing at any point and so was not linked to any specific context.

A binomial logistic regression that included home-unit, trial type and brain region as fixed predictors revealed important differences. For monkey PL, during sham stimulation sessions, there was no notable difference between Blue and Yellow trials conducted in either the Yellow home-unit ($ß = - 0.21 \pm 0.13$, $z = - 1.59$, $p = 0.111$, Figure 5.6E) or the Blue home-unit ($ß = - 0.037 \pm 0.13$, $z = - 0.29$, $p = 0.77$, Figure 5.6E). However, following anterior hippocampal stimulation, performance on Yellow trials in the Yellow home-unit was significantly better than on Blue trials ($ß = 0.83 \pm 0.18$, $z = 4.59$, $p < .001$, Figure 5.6E) when performed in the Yellow home-unit and performance on Blue trials in the Blue home-unit was also significantly increased ($ß = 1.06 \pm 0.18$, $z = 6.02$, $p < .001$, Figure 5.6E).

Monkey MC displayed a similar pattern, though Blue trials were performed significantly better than Yellow trials during sham sessions in both the Yellow home-unit ($ß = 0.42 \pm 0.17$, $z = 2.43$, $p = 0.015$. Figure 5.6F) and in the unfamiliar home-unit ($ß = 0.51 \pm 0.25$, $z = 2.05$, $p = 0.041$, Figure 5.6F), suggesting a potential personal bias for Blue

context trials. Remarkably, after anterior hippocampal stimulation, performance on Yellow trials in the Yellow home-unit improved significantly compared to Blue trials (ß = 0.68 ± 0.14, z = 4.96, p < .001, Figure 5.6F). However, anterior hippocampal stimulation did not affect performance in the unfamiliar home-unit (ß = - 0.067 ± 0.41, z = 0.16, p = 0.870, Figure 5.6F). Considering anterior hippocampal stimulation enhanced performance when the context matched the familiar home-unit environment in which the trials were previously taught, indicates that the anterior hippocampus was responsible for greater context awareness. Specifically, this suggests that context processing in the anterior hippocampus extends beyond the abstract colour cues on the screen to encompass larger, spatially defined environments, as is seen in rodent studies.

Further analysis examined the interaction between brain region stimulation and performance on familiar versus unfamiliar trials for both macaques (Figure 5.7). For monkey PL, there was no difference between sham and pHPC stimulation sessions when compared for familiar and unfamiliar trials (Figure 5.7A). Monkey MC showed similar results (Figure 5.7B), with no significant differences across all brain regions and sham for familiar trials. For unfamiliar trials, posterior hippocampal stimulation showed a trend toward lower performance compared to sham, though it did not reach statistical significance. This may be due to Monkey MC's more varied training experience, due to difficulty learning the individual sequences, and reduced the impact of posterior hippocampal stimulation in remembering precise, spatial layouts as a result.

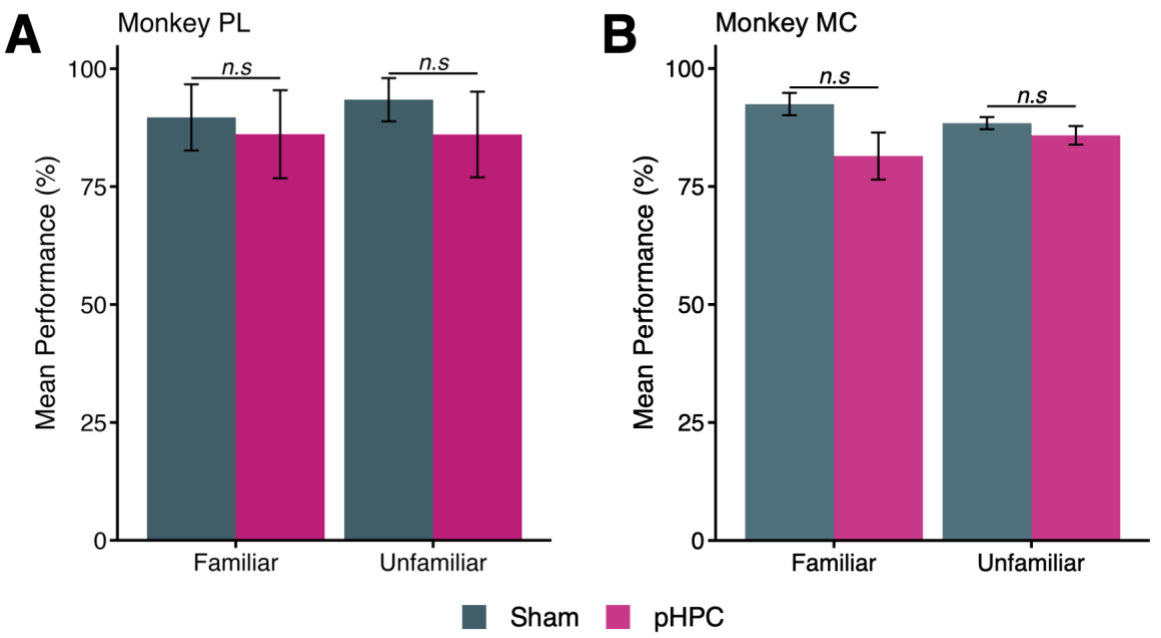

Figure 5.7. Coefficient estimates for performance on familiar and unfamiliar trials following stimulation to the three brain regions and sham.

A) *Shows performance for monkey PL for familiar and unfamiliar trials. Familiar trials correspond to stimuli arrangements onscreen previously experienced during training whereas unfamiliar trials involve arrangements that are novel to the animal.* ***B)*** *Shows performance of monkey MC for the same trial types. Error bars represent standard error around estimated means. Sham relates to sham stimulation sessions and pHPC refers to posterior hippocampal stimulation sessions. Significance is indicated by n.s ($p > .05$).*

5.4. Discussion

This study investigated the effects of low-intensity transcranial ultrasound stimulation (TUS) on context-dependent sequence learning in two rhesus macaques, targeting the prefrontal-hippocampal circuit, due to its known involvement in this type of memory. By leveraging the ability of TUS to modulate neuronal activity with high spatial specificity, the aim was to examine its effect on memory following stimulation of distinct brain regions present within the prefrontal-hippocampal circuit. Namely, the focus was on the anterior and posterior hippocampus (aHPC and pHPC respectively) and the medial prefrontal cortex (mPFC).

Region-specific effects of TUS were found with aHPC stimulation improving performance when the context was stable but impairing performance during unstable context conditions. mPFC stimulation improved performance exclusively in unstable contexts when context was unpredictable and pHPC stimulation showed inconsistent effects, with a potential trend toward impairment throughout.

The initial phase of the experiment involved sequence learning under stable context conditions, where contextual cues (e.g., spatial location of trials) were consistent. Specifically, Yellow context trials were always conducted on the left-side of the home-unit, and Blue context trials were always conducted on the right, enabling the animals to form a clear representation of where to expect which sequence. Following TUS, the aHPC showed to improve performance for both macaques, but neither the pHPC nor mPFC stimulation had an effect. Stimulation of the pHPC showed a trend toward impairing performance in one macaque, though this was not statistically significant for the other.

The differential impact of aHPC versus pHPC stimulation might be a consequence of the distinct functional specialisation seen along the longitudinal hippocampal axis, and each region's individual role in memory processing. Following research across a variety of species, activation of the aHPC is thought to be involved in processing coarse-grained or 'global' information, whereas activation in the pHPC reflects processing of more fine-grained or 'local' information (Poppenk et al., 2013). As such, priming of expected sequences may explain the context-dependent effects of TUS, as outlined below.

In stable contexts, stimulation of the aHPC likely enhanced the macaques' ability to associate broader spatial cues (e.g., left or right home-unit) with the corresponding sequence. For example, entering the left home-unit, which through training was associated with only Yellow context trials, may have primed the Yellow sequence (e.g., object A followed by B). This observation aligns with findings from Smith and Bulkin (2014), who note that revisiting a familiar context can prime relevant memories and enhance retrieval of said memories.

Under unstable contexts, however, where spatial locations were no longer consistently linked to one particular sequence, aHPC priming became counterproductive. Performance now depended on a more specific contextual cue – namely, the screen's background colour. In such cases, aHPC stimulation impaired performance. Notably, despite this overall impairment, performance improved in trials where the first background colour matched the original training conditions (e.g., a yellow background in the left home-unit). This suggests that the aHPC is responsible for processing the contextual information of the trial possibly through a more generalised approach of associating the broader spatial location with the correct sequence. These findings further identify the role of the aHPC in processing the context as a 'global' rather than a 'local' concept.

Similarly, pHPC stimulation may also have been priming except on a much smaller scale. Activity in the human pHPC has been closely tied to spatial cognition (Duncan et al., 2012; Strange et al., 1999) with selective activation being shown during the retrieval of spatial information of episodic memories (Hoscheidt et al., 2010). Indeed, when participants are asked to recall local spatial details from past life events, such as their wedding seating plan, greater activation was seen in the pHPC than in the aHPC (Nadel et al., 2013). As such, in this study, pHPC stimulation may have primed fine-grained spatial details, such as the specific configuration of objects onscreen, rather than the sequential order of objects.

During initial learning, both animals were trained on a set number of trials with fixed object-location sequences. These trials were later used in the testing phase, along with trials where the objects were arranged in novel, previously unseen configurations, yet still uniformly arranged on the screen. As the novel configuration of the objects in both contexts outweighed familiar ones during testing, the priming effect created as a

result of pHPC stimulation may have resulted in greater mismatch and overall performance impairment due to expectation of an object in a position it no longer occupied. The exact mechanisms underlying these effects remain speculative and warrant further exploration.

Improved performance following mPFC stimulation during unstable contexts supports its role in resolving ambiguity from overlapping memories and coordinating with the hippocampus to facilitate retrieval of correct context-dependent sequences. Prior research has shown that the mPFC aids in distinguishing overlapping contexts and guiding hippocampal activity (Navawongse & Eichenbaum, 2013; Preston & Eichenbaum, 2013). In the present study, unstable contexts introduced considerable overlap between the two learned sequences, particularly after relearning altered the original object-context associations. The mPFC likely helped resolve this conflict by directing hippocampal processes to disambiguate competing sequences and guide the correct temporal response. This may have been achieved through the use of mental schemas, where the associations between each learned object could shift as new sequence orders are reinforced.

During relearning, mPFC stimulation further enhanced performance, consistent with its involvement in adaptive forgetting mechanisms that suppress irrelevant or competing memories (Milivojevic et al., 2015). Interestingly, aHPC stimulation also improved memory performance despite the reversed object-context sequences. This result seems counterintuitive, as stimulation of the aHPC should theoretically impair performance by reinforcing previously learned (but now incorrect) associations. One possible explanation is that previously learned spatial cues (e.g., Yellow context in the left home-unit) remained consistent and enabled the aHPC to prime the global contextual associations. These associations may have provided a foundation for the mPFC to refine and apply to the correct sequences, further highlighting the possibility of schemas within the mPFC.

These findings highlight distinct and complementary roles of the aHPC, pHPC, and mPFC in context-dependent sequence learning. The aHPC appears to support global context associations, while the pHPC facilitates fine-grained spatial processing. The mPFC is responsible for resolving contextual ambiguity and suppressing irrelevant memories.

The effects of TUS on cognitive performance observed in this study align with prior evidence from *in vivo* studies conducted in both animal models and humans. The impact of TUS – whether excitatory or inhibitory – varies depending on experimental parameters and the regions targeted. Studies using TUS as a neuromodulatory tool in rodents have demonstrated enhanced memory performance following a single application of ultrasound (Blackmore et al., 2023). For instance, in Alzheimer's disease model mice, TUS applied to the hippocampus alongside microbubble injections improved spatial memory, as evidenced by enhanced performance in the Morris Water Maze (Shen et al., 2020). Similar results were reported in dementia model Sprague-Dawley rats, where TUS treatment co-delivered with microbubbles enhanced spatial memory likely through upregulation of brain-derived neurotrophic factor (BDNF), a key mediator of neurogenesis and synaptic plasticity (Shin et al., 2019).

In contrast to these studies, the research presented in this chapter utilised TUS solo. Microbubbles facilitate blood-brain-barrier opening, amplifying certain neurotrophic effects, but their absence in this study suggests that the observed improvements were more likely due to direct neuronal stimulation. Supporting this hypothesis, TUS has been shown to increase dendritic spine density, elevate the expression of GluN2A subunits of glutamate receptors, and enhance the frequency of spontaneous excitatory post-synaptic currents following repeated hippocampal stimulation over 10 days in rats (Huang et al., 2019). Furthermore, in a human study, hippocampal stimulation with TUS in Alzheimer's patients, improved recognition memory and enhanced glucose metabolism all with the absence of blood-brain-barrier opening (Jeong et al., 2022). These findings point to a direct, localised neuromodulatory effect of TUS.

Several molecular mechanisms have been proposed to account for the effect of TUS on neuronal activity, with one prevailing hypothesis focusing on the influence of mechanical ultrasound pressure waves on cell membranes (Yoo et al., 2022). These waves are thought to modulate ion channel gating resulting in the activation of sodium and calcium channels and consequently increasing neuronal excitability. Notably, in hippocampal slice cultures, TUS has been demonstrated to stimulate neuronal activity in CA1 pyramidal neurons via this mechanism (Tyler et al., 2008).

In addition to increasing excitability, the specific TUS protocol employed in this study may have entrained neurons to a theta rhythm. The pulse repetition interval (100

milliseconds, corresponding to a frequency of 10 Hz) falls within the theta rhythm frequency range for primates of around 4-12 Hz (Colgin, 2012; Watrous et al., 2013). Theta rhythms are important for coordinating activity across brain regions and are associated with improved timing of neuronal firing, enhanced functional connectivity, and optimised information transfer. Such entrainment could explain some of the performance enhancements observed, particularly in tasks requiring context integration.

Theta oscillations are fundamental to prefrontal-hippocampal interactions and cognitive processes such as memory retrieval and decision making (Eichenbaum, 2017). Improved synchrony between theta and gamma oscillations in this network facilitates these processes by enhancing temporal coordination and functional connectivity. For example, Roberts et al. (2018) demonstrated that rhythmic auditory and visual stimulation, designed to entrain theta oscillations, improved source memory performance in humans. This improvement persisted into post-entrainment memory-retrieval tasks, suggesting that theta modulation has lasting effects on cognitive performance. Similarly, TUS-induced theta entrainment in the present study likely enhanced synchrony throughout the prefrontal-hippocampal circuit, particularly during the unstable context condition when communication between the aHPC and mPFC was important for relaying information regarding the abstract contextual information, particularly when spatial cues became less reliable.

The observed effects may also involve intermediary structures in the prefrontal-hippocampal network, such as the entorhinal cortex. The entorhinal cortex is a bidirectional interface between the hippocampus (e.g., the dentate gyrus and CA3) and the mPFC, and has been implicated in spatial memory. Indeed, deep-brain stimulation of the entorhinal cortex has been shown to rescue spatial memory in a mouse model of Alzheimer's disease (Mann et al., 2018). Given the anatomical proximity of the aHPC to the entorhinal cortex, it is plausible that a portion of the entorhinal cortex was indirectly stimulated during aHPC-targeted TUS sessions. This inadvertent stimulation may have engaged mechanisms intrinsic to the entorhinal cortex, facilitating the coordination of spatial and contextual information. Such involvement could explain aspects of the observed performance enhancement, particularly during conditions where global context integration played a prominent role. This potential contribution of the entorhinal cortex highlights the interconnected nature

of the prefrontal-hippocampal circuit and suggests that broader network effects may underlie the region-specific outcomes of TUS. Indeed, there is strong evidence from MRI data collected from macaques that TUS affects the functional connectivity of the stimulated region with its associated areas throughout the brain (Verhagen et al., 2019; Folloni et al., 2019).

While this study provides compelling evidence for region-specific effects of TUS on context-dependent sequence learning, several limitations must be acknowledged. First, the small sample size of two macaques limits the generalisability of these findings. Though consistent trends were observed, larger cohorts are necessary to validate these effects across a broader population, especially if TUS is to be translated into a therapeutic tool in humans. Including additional species with varying prefrontal-hippocampal connectivity could also provide greater insight into the generality of TUS effects. For example, studies involving rodents or marmosets could offer advantages due to the feasibility of larger sample sizes, enabling more robust statistical analyses. Although, as highlighted by the results in Chapter 2 and 3, the task complexity achieved by macaques in this study might be challenging to replicate in other species.

The main advantage of working with other species, particularly smaller mammals, would be the increased experimental control over context (i.e., manipulation of the spatial surroundings would be easier). This in turn will help to disentangle the roles of the specific regions in the prefrontal-hippocampal network when combined with TUS, providing a clearer picture of the circuit's functional architecture.

Second, the absence of direct electrophysiological recordings during TUS sessions limits the ability to confirm hypothesised neural mechanisms, such as theta entrainment or synaptic activity changes. Employing *in vivo* electrophysiological recordings or advanced imaging techniques, such as calcium imaging or fMRI, during TUS would provide direct evidence for these mechanisms. Furthermore, such approaches could elucidate the precise neural dynamics driving the observed behavioural changes and also give a timeframe as to when and for how long the neuromodulatory effects were present.

In summary, this study demonstrates the potential of TUS as a powerful tool for modulating neural circuits underlying context-dependent sequence learning. The findings highlight the differential roles of the anterior hippocampus (aHPC), posterior

hippocampus (pHPC), and medial prefrontal cortex (mPFC): the aHPC supports global context integration, the pHPC contributes to fine-grained spatial processing, and the mPFC resolves contextual ambiguity. These results align with previous research across many species on the functional specialisation of these regions and also provide novel insights into the potential of TUS as a non-invasive neuromodulation technique.

With its ability to precisely and non-invasively target neural structures deep within the brain, TUS holds promise not only as a research tool in cognitive neuroscience but also as a potential therapeutic intervention for memory-related disorders. Further refinement of experimental methods – such as the incorporation of neural recordings – is essential to maximise its translational value. Future studies can build on these promising results to further our understanding of brain function and expand the application of TUS to a clinical setting.

Chapter 6: General Discussion

The overarching aim of this thesis was to investigate the ability of different species to learn context-dependent sequences. By exploring both species-specific differences and shared capacities in this type of learning, the research provides valuable insights into the mechanisms underlying episodic-like memory – a form of episodic memory proposed to exist in nonhuman animals.

6.1. Species-Specific Adaptations in Context-Dependent Sequence Learning

In Chapter 2, rats were tested for their ability to learn and recall pairs of objects whose order depended on the context in which the trial began. While the rats successfully recalled object sequences when the context remained consistent throughout a trial, they faced significant difficulties when sequences spanned multiple contexts. Among the cohort, only two rats demonstrated the ability to flexibly switch between static context sequences and overlapping context sequences. These findings indicate that, although the capability to handle multiple context-dependent sequences exists, it is both rare and requires prolonged learning to emerge.

Chapter 3, adapted the paradigm above to test marmosets, introducing a key modification: all objects from both contexts were present in each phase of the trial. This increased the potential for memory interference, even in single context trials. While all marmosets successfully learned these single context sequences, they struggled to adjust when the context shifted mid-trial. Nevertheless, marmosets adapted more readily than rats during the learning phase, though they still exhibited difficulty in fully grasping the task's demands. The increased interference likely added an additional layer of complexity, as numerous cues were common to both contexts, thereby highlighting the cognitive challenge posed by overlapping contextual features.

In Chapter 4, the investigation was extended to macaques, employing a more sophisticated setup that replaced the physical maze and tangible objects in the previous two experiments with touchscreens integrated into the animal's home-units. Context was represented both physically, as the spatial location within the home-unit, and abstractly, as the background colour displayed on the touchscreen. This novel approach created an opportunity to explore neuromodulatory effects, enabling an investigation of how targeted transcranial ultrasound stimulation influenced the prefrontal-hippocampal circuit, as discussed in Chapter 5.

Macaques displayed a marked ability to learn context-dependent sequences, regardless of whether objects were randomly rearranged between trials. Furthermore, they adeptly handled mid-trial contextual switches, maintaining high performance throughout. This exceptional adaptability allowed for further assessment into the influence of transcranial ultrasound stimulation (TUS) on memory retrieval processes. Stimulation of the anterior hippocampus enhanced performance in single context trials, whereas stimulation of the medial prefrontal cortex improved performance during trials involving mid-trial contextual shifts. These results highlight distinct functional contributions of the prefrontal-hippocampal circuit to context-dependent sequence learning and retrieval.

Collectively, these findings shed light on the cognitive and neural mechanisms underlying context-dependent learning and provide comparative insights into episodic-like memory through three key animal models widely used in neuroscience.

Across all three species, learning single context sequences – where the context remained stable during object selection – was relatively straightforward. Rats generally performed well under these conditions but exhibited notable difficulties when the context varied from trial to trial. This was surprising, as although rats experienced both context-specific sequences during this phase, each individual trial featured only a single context and its corresponding object pair, thereby limiting the potential for within-trial interference (Section 2.3.1). Error analyses revealed that the rats frequently struggled with the first object in a sequence, often perseverating by repeating the movement executed at the end of the previous trial, such as consistently choosing the object on the right side. This pattern suggests that rats relied on simpler egocentric movement strategies, which proved less effective when contexts were less stable.

Marmosets exhibited high accuracy in learning single context sequences, even in the presence of increased interference caused by the availability of all objects in each trial (Section 3.3.1). Marmosets effectively inhibited the irrelevant associations and achieved threshold performance in a short time scale. Similarly, macaques demonstrated robust performance in single context (or stable context) trials (Section 4.3.1), even when objects were presented in randomised configurations across trials, suggesting both species had capacity in adapting to context-dependent object sequences.

While single context trials primarily required animals to rely on consistent contextual cues to guide object selection, two context trials introduced an additional layer of complexity. These trials required animals to disambiguate competing information and adapt to rule changes mid-trial, reflecting greater demands on cognitive flexibility and memory processes. As such, performance during two context trials varied significantly across species.

Rats struggled considerably during two context trials, with only a few individuals achieving the desired criterion (Section 2.3.2). These difficulties appeared to stem from their failure to adapt to the context-dependent changes in object-order. Specifically, the correct object choice was not based on a fixed sequence across trials but depended on the temporal order in which each context was entered. For example, an object might be correct when a context is encountered first but incorrect if the same context is visited second within a testing session. This subtle temporal rule posed a significant challenge. As in the previous phase, rats exhibited a pronounced egocentric bias, favouring recently visited or non-depleted object locations over the application of the contextual rule. Additionally, interference from previously learned object-context associations may have further disrupted the integration of the new, context-contingent sequences. Together, these behaviours indicate a continued reliance on simpler, habitual strategies that were ill-suited to the more flexible, hierarchical demands of the two context condition.

Marmosets also found two context learning challenging, however, this may have been partially attributed to the design of the apparatus, which appeared to reduce their motivation to engage with one of the contexts (Section 3.3.7). Marmosets consistently displayed a preference for the brighter context over the darker one, suggesting an ecological bias. This behavioural tendency is consistent with previous findings – for example, Mendes & Huber (2004) reported that marmosets exhibited persistent preferences for particular testing environments during an object permanence task, indicating stable individual or species-level biases that may influence task performance.

Macaques exhibited the greatest flexibility and adaptability during two context trials, quickly relearning sequence-context associations (Section 4.3.2) and successfully

adapting to mid-trial contextual shifts (Section 4.3.3). This adaptability highlights their capacity to manage increased task complexity and cope with competing information.

The flexibility the macaques exhibited likely reflects the demands of two context learning, which introduced increased task ambiguity and interference for all species. Successfully navigating these conditions requires animals to adopt more sophisticated strategies for recall, transitioning from rigid, rule-based memory processes to more flexible forms of categorisation (Section 2.4). This shift is hypothesised to engage the prefrontal cortex, particularly in resolving potential ambiguity when similar information can appear across multiple contexts (Easton & Gaffan, 2002; Gaffan et al., 2002). While performance on single context sequences may rely predominantly on hippocampal-dependent memory processes, the additional demands of switching contexts – and effectively rules – mid-trial necessitate the integrative involvement of the prefrontal cortex.

6.2. Neural Mechanisms Underlying Contextual Flexibility

Evidence from the rodent literature supports this interpretation. Object-context paradigms, which require animals to distinguish between familiar objects based on the surrounding context, highlight a required inclusion of the prefrontal cortex. For example, inactivation of the medial prefrontal cortex increases errors in judgement about object-location associations, indicating that this region is important for the retrieval and selection of appropriate object-context memories (Navawongse & Eichenbaum, 2013; Place et al., 2016; Barker & Warburton, 2020; Malik et al., 2022). Specifically, during medial prefrontal inactivation, neurons in the hippocampus that otherwise signalled only one of the two items in a given context came to signal both. This finding aligns with broader theories that the prefrontal cortex exerts top-down control by inhibiting irrelevant memories (Depue, 2012) and subsequently biasing the retrieval of context-appropriate memories (Miller, 1999). The same inactivation also impaired task performance, further indicating that top-down bias from the medial prefrontal cortex is important for successful behaviour.

Anatomical differences between species provide additional insight into why rats experienced greater challenges during two context learning. As discussed in Chapter 1.4, there is ongoing debate regarding the homology of the medial prefrontal cortex across rodents, nonhuman primates, and humans. In particular, rats lack a granular

prefrontal cortex which is present in primates, including marmosets and macaques (Burman et al., 2006). The controversy, however, centres not on the presence or absence of this region, but rather whether the rat prefrontal cortex functions as a 'replica-in-miniature' version of the primate prefrontal cortex (Kolb, 2007). Behavioural similarities following lesions lend partial support to this idea. For example, lesions in the medial prefrontal cortex impair learning in delayed response tasks in rats (Kolb et al., 1974), just as lesions in the granular prefrontal cortex impair these tasks in primates (Goldman et al., 1971). And yet, impairments in similar tasks are also observed following lesions to agranular regions in primates such as the anterior cingulate cortex or prelimbic cortex (Meunier et al., 1997). This suggests that the rodent medial prefrontal cortex is, if anything, functionally analogous rather than homologous to the primate prefrontal cortex. The consensus that prevails, however, is that the granular prefrontal cortex, is unique to primates and represents distinct evolutionary advancements (Preuss & Wise, 2022).

In both rats and primates, the medial prefrontal cortex plays a role in decision-making based on predicted outcomes. This region enables animals to choose actions linked to specific outcomes or to flexibly switch behaviours when circumstances change, without directly generating motor commands (Murray et al., 2011). This system likely evolved in early mammals to promote rapid behavioural adjustments in more dynamic environments. In its absence, animals default to the strongest pre-existing associations or inherent tendencies, which only adapt slowly over time.

Behavioural studies in rats illustrate this mechanism. For example, rats can learn a matching-to-position task, where they must return to a location where they previously received food (Marighetto et al., 1998). To perform this task successfully, rats must overcome an innate tendency to explore foraging sites that they have not exploited recently and avoid those that they have. While intact rats typically master this task within 15-20 training sessions, lesions in the prelimbic and infralimbic regions of the medial prefrontal cortex significantly slow learning (Dias & Aggleton, 2000). These findings suggest that the medial prefrontal cortex enables animals to overcome prepotent tendencies, facilitating more rapid behavioural adjustments with fewer errors, further enhancing behavioural flexibility.

This dynamic may explain why rats struggled with two context learning. Their difficulty in adapting to the temporal order of objects during context shifts likely stemmed from interference caused by stronger pre-existing object-context associations learned during the single context phase. Furthermore, when contexts changed across trials, rats often demonstrated an egocentric movement bias, consistently opting to visit non-depleted locations rather than adhering to task rules. This suggests they are acting on their innate tendency to avoid recently foraged sites (described above). In natural environments, such behaviour could be advantageous, as it promotes exploration of unexploited foraging sites, increasing likelihood of finding food, however, in the experimental setting, this tendency conflicted with task demands, leading to increased errors.

Throughout the thesis, the focus was on the medial prefrontal cortex as it is a region with structural and functional consistencies across all three species, however, differences in more anterior prefrontal morphology likely contributed to the observed variations in learning strategies and performance among the three species.

Marmosets, as New World primates, occupy an intermediate position between rats and macaques. While they possess granular prefrontal regions, these are less subdivided and less organised than in macaques (Burman et al., 2006; Burman & Rosa, 2009). In addition, the density of pyramidal cells in the human prefrontal cortex is approximately four times greater than in marmosets, and the granular prefrontal cortex occupies less than 10 % of the total brain volume in marmosets compared to 30 % in humans (Elston, 2003). These morphological differences, are believed to reflect potential cognitive limitations, including reduced working memory (Schaeffer et al., 2019).

This has since been shown to limit marmosets in a non-spatial working memory task to monitor a maximum of two items in working memory (Zlatkina et al., 2024), compared to macaques who are able to monitor as many as five items in a similar task (Petrides, 2000). The authors attest the limited ability to the marmosets lacking a broader area 46 (dorsolateral prefrontal cortex) as is seen in macaques (Burman et al., 2006; Zlatkina et al., 2024). Importantly, previous studies have demonstrated that marmosets are able to maintain information in recognition memory with delays of up to 16 seconds (Nakamura et al., 2018). This suggests that it is the number of stimuli

within working memory that is the limiting factor rather than the length of time a particular stimulus is held in memory.

Marmosets succeeded in learning single context sequences, despite the presence of available objects, which created heightened interference and overlap between contexts. Their ability to inhibit such interference and achieve threshold performance suggests a possible involvement of more than just the medial prefrontal cortex. Specifically, the lateral regions of the prefrontal cortex – which are granular and absent in rats – may assist the medial prefrontal cortex in biasing memory retrieval effectively.

In terms of connectivity, the lateral prefrontal cortex does not directly innervate the hippocampus or medial temporal lobe. Instead, it forms bidirectional connections with the medial prefrontal cortex, namely area 32, also called the anterior cingulate cortex (Barbas & Pandya, 1989; Anderson et al., 2016). This indirect connectivity likely enhances the medial prefrontal cortex's ability to bias memory retrieval and manage task complexity. The absence of a granular prefrontal cortex may thus have contributed to rats' poor performance in two context sequence learning.

Studies involving context-to-goal mapping reinforce this idea. In monkeys, lesions to granular prefrontal regions, specifically, ventral and orbitofrontal areas, significantly impair the ability to learn context-goal mappings quickly, resulting in considerably longer learning (Bussey et al., 2001). It should be noted that, monkeys do eventually learn these associations implying that while the granular prefrontal cortex is important for efficient learning, other regions can compensate, albeit less effectively. This parallels the observation that some rats succeeded in the two context task, likely relying on compensatory mechanisms (i.e., purely the medial prefrontal cortex) while others failed entirely.

It is important to consider that the object pair timing — that is, the delay between the first and second object presentations in a trial — varied across species and between task phases, which could have contributed to differences in performance. In rats, this delay was longest during the two context phase, as animals had to traverse a lengthy corridor and transition into a new spatial context before encountering the second object. Marmosets also experienced delays between object presentations, though these were notably shorter in the single context condition and relatively modest in the two context phase due to the smaller apparatus size. In contrast, macaques

experienced the shortest and most consistent intra-trial intervals, with object pairs presented sequentially within the same viewing session regardless of condition. These differences in temporal structure may have influenced memory demands, susceptibility to interference, or the reliance on working memory versus long-term representations across species.

6.3. Implications for Episodic-Like Memory and Comparative Cognition

The specific roles of the hippocampus and prefrontal cortex in episodic-like memory remain uncertain. It is not fully understood whether one region predominantly stores memories, whether both contribute equally, or if their roles depend on the context of the memory task. Evidence suggests that hippocampal activity is indispensable for memory retrieval, even after prolonged periods of memory consolidation. For example, retrograde amnesia persists for contextual memories even after 100 days, a period sufficient for potential transfer to other brain regions (Broadbent & Clark, 2013; Clark et al., 2005).

Intriguingly, studies on adult hippocampal neurogenesis further highlight its role in memory retrieval and maintenance. Increased neurogenesis following learning has been shown to impair memory retrieval (Akers et al., 2014), while impairing neurogenesis preserves context-dependent neural patterns in the dentate gyrus (Denny et al., 2014). These findings suggest that the hippocampus serves as more than a passive storage site; it actively maintains and retrieves contextual memories, regardless of its interactions with other brain regions.

Beyond memory retrieval, the hippocampus also appears to be involved in prediction and planning. Studies show that hippocampal activity predicts anticipated stimuli (Jafarpour et al., 2017), future paths during spatial navigation in rodents (Dragoi & Tonegawa, 2011), and imagining future events in humans (Addis & Schacter, 2008). This predictive function may explain why TUS of the anterior hippocampus enhanced macaque performance during single context trials during the stable context condition (Section 5.3.1). By increasing activity in hippocampal regions that guide contextual processing, TUS may have enhanced sequence expectations, leading to better performance.

This predictive role, however, can also be disruptive under conditions of contextual ambiguity. For example, stimulation of the anterior hippocampus during two context

trials in the unstable context condition impaired performance (Section 5.3.3), particularly when the background colour of a trial conflicted with the physical surroundings where the task was originally learned. If the hippocampus primes an expected context, heightened activity induced by TUS may amplify this effect, hindering performance when contextual cues are incongruent. Moreover, object representations in the hippocampus are perceived to be secondary to location representations (Manns & Eichenbaum, 2009). This suggests that if TUS enhances hippocampal persistence of a specific spatial context (i.e., the home-unit side), the medial prefrontal cortex may face greater difficulty overriding this effect to manage contextual ambiguity effectively.

Conversely, stimulation of the medial prefrontal cortex enhanced performance during two context trials, particularly under conditions of contextual ambiguity (Section 5.3.3). This finding highlights the role of the medial prefrontal cortex in managing competing contextual information and guiding adaptive behaviour. By exerting top-down control, the medial prefrontal cortex may resolve conflicts between competing contextual cues, enabling macaques to adapt more effectively to rule changes and ambiguous task conditions.

Unlike the hippocampus, which prioritises detailed contextual information, the medial prefrontal cortex supports the development and maintenance of schemas – generalised frameworks derived from regularities across a range of experiences rather than specific episodic details (McClelland et al., 1995). These schemas allow flexible application of knowledge across diverse contexts, enabling behaviour to transcend reliance on specific episodic memories.

Humans, with their highly lateralised prefrontal cortex, show heightened schema-based flexibility. Research indicates that context shifts, or event boundaries disrupt memory, but strategies such as mentally transforming oneself can reduce context-dependent forgetting (Masicampo & Sahakyan, 2014). Notably, within-context sequential learning in humans has been associated with increased medial prefrontal cortex activity, specifically in the ventromedial portion, but not for sequences that spanned a boundary. Instead, across-context associations were associated with increased activation in the lateral prefrontal cortex (DuBrow & Davachi, 2016).

Considering for the rats that sequence learning required them to traverse in and out of a context for every decision made, this may have implicitly biased learning. For instance, if lateral prefrontal regions are critical for tracking temporal changes in sequences across boundaries, introducing a delay or gap between objects could have increased the task's reliance on these areas. Furthermore, in humans walking through a doorway in a virtual environment has been associated with forgetting (Radvansky & Copeland, 2006). It is possible that rats struggled to associate the two contexts as part of unified trial or to retain prior actions across these transitions.

When sensory cues shift abruptly while the memory goal remains unchanged, the hippocampus and lateral prefrontal cortex may help organise information that spans the change (DuBrow & Davachi, 2016), maintaining an internal context to bridge the discontinuity (Polyn & Kahana, 2008). Koechlin et al. (2000) similarly suggest these regions may specialise in handling sequences across unpredictable events, whereas the medial prefrontal areas respond to predictable sequences. Thus, the lateral prefrontal cortex may have evolved in humans to sustain goal-orientated behaviour over increasingly distant timeframes, reflecting an adaptation for managing complex, temporally extended contexts.

6.4. Strengths and Limitations

A major strength of this thesis, often a limiting factor in comparisons between rodent and primate studies, is the minimal restriction on self-motion, particularly during navigation-based memory tasks (Thome et al., 2017). Restricting self-motion reduces the number and specificity of active neurons in the rodent hippocampus (Terrazas et al., 2005), potentially altering behavioural responses seen. Although the macaques did not tangibly interact with objects, they were required to move within their home-unit to complete the task. Notably, one macaque progressed through the entire experiment, moving between screens every five trials, while the touchscreens remained consistent to a specific side of the home-unit. The other macaque, though less motivated, was rigorously tested under matched contextual and spatial conditions. This unrestricted movement likely contributed to the robust effect observed during transcranial ultrasound stimulation of the anterior hippocampus – an effect that may have been absent if the animals were restrained.

Similarly, for the rats and marmosets, avoiding use of touchscreens and employing maze-based tasks enabled more equitable comparisons. A limitation, however, of the apparatus, specifically for the marmosets, was the absence of a starting corridor, which the rats' maze included. This omission meant the marmosets completed the sequence in a single motion, unlike the rats, which navigated in and out of the contexts to complete the sequence. Considering the potential impact of naturally occurring environmental boundaries negatively affecting event segmentation and forgetting, this likely made the task more challenging for the rats. Additionally, introducing a neutral corridor for the marmosets, allowing entry into both contexts through this space rather than a tunnel that went through the middle of the contexts, could improve motivation and align the task structure more closely with that of the rat experiment.

6.5. Future research directions

Future studies should aim to address the limitations highlighted in this research to expand our understanding of the region-specific effects of transcranial ultrasound stimulation on context-dependent sequence learning. Increasing the sample size, particularly in macaques, is a critical next step to ensure the generalisability of these findings and to validate their consistency across broader populations. Comparative studies involving species with varying prefrontal-hippocampal connectivity, such as rodents or marmosets, could also provide additional insight into the applicability of TUS across species. In particular, these smaller species offer the advantage of larger sample sizes and greater experimental control over environmental contexts, enabling more nuanced manipulations of spatial surroundings. The complexity of the tasks used, however, will need to be adapted both cognitively and ecologically for the species being tested.

Additionally, incorporating direct neural measurements during TUS sessions is imperative to confirm the hypothesised mechanisms driving behavioural changes outlined in this thesis. Techniques such as *in vivo* electrophysiological recordings, optogenetics, calcium imaging, or functional MRI could provide real-time insights into neural dynamics, such as theta entrainment or synaptic activity changes, and establish the temporal characteristics of TUS-induced effects. These approaches would also help delineate the roles of the anterior hippocampus, posterior hippocampus, and

medial prefrontal cortex in sequence learning, further clarifying the functional architecture of the prefrontal-hippocampal network.

By combining these methodological refinements with cross-species comparisons, future research can enhance our understanding of TUS as both a neuroscientific tool and a potential therapeutic intervention. Such efforts will be essential for translating the findings into clinical applications for treating memory-related disorders, maximising the translational impact of this promising neuromodulation technique.

6.6. Conclusion

This thesis provides a comparative investigation into context-dependent sequence learning across three species — rats, marmosets, and macaques — offering insight into the cognitive, behavioural and neural mechanisms underlying episodic-like memory. Rats and marmosets were proficient in learning fixed context-dependent sequences but struggled with sequences requiring mid-trial context shifts, whereas macaques demonstrated flexibility in mastering both types of sequence. These results underscore an evolutionary gradient in the ability to integrate contextual and temporal information, likely reflecting differences in neural architecture.

Using transcranial ultrasound stimulation in macaques, the research identified a dissociation between the hippocampus and prefrontal cortex. Modulation of the anterior hippocampus enhanced performance in sequences requiring stable context integration, while prefrontal cortex stimulation improved performance particularly during trials with unpredictable context shifts. These findings suggest that the effects of stimulation may not differ in relation to the early versus late stages of training, but rather are more pronounced during phases involving dynamic, unpredictable context changes. This highlights the distinct yet complementary roles of these regions in context-dependent learning.

Overall, this work deepens our understanding of episodic-like memory by linking behavioural flexibility to neural mechanisms, revealing both conserved and divergent pathways across species, and providing insights into the evolution of memory systems tailored to dynamic environments.

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