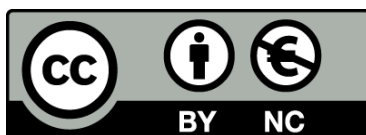




UNIVERSITAT<sub>DE</sub>  
BARCELONA

## Role of prefrontal monoamines in a novel translational test of working memory for rodents

Begoña Gamallo Lana



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PhD Program in Biomedicine  
University of Barcelona

## **Role of prefrontal monoamines in a novel translational test of working memory for rodents**

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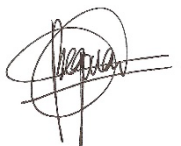
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The present work has been supported by the following projects:

1S10OD023675 (NIH S10). Bussey-Saksida Touch Screen System. Contact PI/Project Leader: MAR, ADAM CHRISTOPHER. Awardee Organization: NEW YORK UNIVERSITY SCHOOL OF MEDICINE.

5U19NS107616 ; Sub-Project ID 9163 (NIH 09/01/23-8/31/28). Oxytocin Modulation of Neural Circuit Function and Behavior. Contact PI/Project Leader: MAR, ADAM CHRISTOPHER. Awardee Organization: NEW YORK UNIVERSITY SCHOOL OF MEDICINE.

I would also like to thank “La Caixa” fellowship for postgraduate studies that supported my postgraduate work that served as ground basis to launch my PhD thesis.

# Acknowledgements

This thesis has been a tremendous labor of love from the very beginning.

I would not be sitting down and writing this document without the continuous support I have received from many people over the years.

First and foremost, Adam and Paco, the most wonderful thesis directors and mentors I could have ever asked for.

Adam, how can I even begin to thank you? You became a strong pillar in my research career the day I met you in Cambridge. Your support throughout the years has been crucial, not only for my development as a young (or not that young anymore) neuroscientist, but also for my development as a human being. Your genuine curiosity for life, for science, your continuous optimism and perseverance are so contagious. Thanks for sharing your wisdom, your kindness and for being an inspiration to me.

Paco – gràcies. Gràcies per tenir la porta sempre oberta i escoltar les meves propostes. Gràcies per estar sempre disposat a ajudar i donar-me suport professional. Gràcies per oferir els teus coneixements i experiència, i fer-ho sempre amb una motivació i ment curiosa per la ciència. L'estada amb l'Anna al teu laboratori va ser la primera que vaig fer en relació amb la neurociència. Els dos sou responsables per haver-me enganxat a aquest camp tant interessant i addictiu.

You both have helped me shape this work into something I am very proud of. I deeply admire both of you, your research contributions to science and all the knowledge you have volunteered with your work. This thesis would most definitely not exist without you.

No hagués pogut caminar aquest camí sense el suport de la família i els amics. Em sento molt afortunada de rodejar-me i comptar amb les persones de la meua vida. Tot i amb un oceà pel mig, hi ha amigues que sembla que estiguin al meu costat cada dia. Vull donar especial menció a l'Alba, Anna, Sandra, Moni, Silvia i Berta per caminar al meu costat, la majoria, des de que vam aprendre a parlar. Gràcies per compartir aquest vincle que no es perd ni amb la distància i que espero no perdre mai. Gràcies també als millors biòlegs humans i metges amb els que he compartit tantes penes i glòries i que tinc la sort de tenir com amics.

También tengo la suerte de contar con una familia muy numerosa y, aún más, maravillosa. Son mi muralla y mi fortaleza, y el mejor equipo que podría pedir para navegar esta vida. Gracias por molar tanto y hacer mi vida más completa y llena de fuerza.

Por último, quiero dedicar esta tesis a mis padres. ¿Qué sería de mí sin vosotros? Creo que no sería capaz de explicar lo que siento por mis padres, aunque me dieran 900 páginas más para escribir. Sois mi luz, mi paz y mi corazón. Sois el amor constante en mi vida que no flaquea y siempre crece. Gracias por vuestro apoyo incondicional, por construir la mejor familia que podría haber soñado y por hacerme reír incluso en los momentos más difíciles. Sois los mejores y os quiero infinito.

Each and every one of you, mentioned here by name or otherwise, have been crucial and represent a key piece to this thesis and in my life. Thank you!

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# 1. Abbreviations

|         |                                                    |
|---------|----------------------------------------------------|
| 5-HT    | Serotonin (5-hidroxitriptamine)                    |
| ADHD    | Attention Deficit-Hyperactivity Disorder           |
| AP      | Anteroposterior                                    |
| ASD     | Autism Spectrum Disorder                           |
| AUC     | Area Under the Curve                               |
| CBTT    | Corsi Block-Tapping Test                           |
| D1R     | Dopamine Receptor D1                               |
| D2R     | Dopamine Receptor D2                               |
| DA      | Dopamine                                           |
| DD      | Delay Discounting                                  |
| DG      | Dentate Gyrus                                      |
| DLS     | Dorsolateral Striatum                              |
| DNMS    | Delay Non-Match to Sample                          |
| DRD     | Delay and Reward Discounting                       |
| DV      | Dorsoventral                                       |
| EOWM    | Experimentally-Ordered Working Memory              |
| g5-HT2h | Green GRAB5-HT2h sensor                            |
| gDA3h   | Green GRABDA3h sensor                              |
| GFP     | Green Fluorescent Protein                          |
| GPCR    | G protein-coupled receptor                         |
| GRAB    | G protein-coupled receptor [GPCR]-activation-based |
| HPC     | Hippocampus                                        |
| mGluR5  | Metabotropic Glutamate Receptor 5                  |
| ML      | Mediolateral                                       |
| mPFC    | Medial Prefrontal Cortex                           |
| OFC     | Orbitofrontal Cortex                               |

|       |                                               |
|-------|-----------------------------------------------|
| PFC   | Prefrontal Cortex                             |
| p.o.  | Oral administration                           |
| rDRD  | Rodent Delay and Reward Discounting           |
| rEOWM | Rodent Experimentally-Ordered Working Memory  |
| rSOWM | Rodent Self-Ordered Working Memory            |
| SOWM  | Self-Ordered Working Memory                   |
| SOPT  | Self-Ordered Pointing Task                    |
| STM   | Short-term Memory                             |
| SWM   | Spatial Working Memory                        |
| TUNL  | Trial-unique, delayed nonmatching-to-location |
| WM    | Working Memory                                |

## **2. Introduction**

### **2.1. Working memory**

#### **2.1.1. Working memory: definition**

Working memory (WM) can be broadly defined as a system or set of processes that are necessary to represent relevant information while performing complex or multistep cognitive tasks. The term emerged in the context of models of human memory which highlighted the importance of a short-term, limited capacity storage mode that functions separately from a more permanent long-term memory store (1, 2). The concept of WM was popularized by Alan Baddeley and colleagues in a model emphasizing the active processing or manipulation of information, beyond its passive storage (3-5). The original WM model proposed that four interrelated components acting on short-term information (3, 4, 6): a “phonological loop” for processing auditory information, a “visuospatial sketchpad” for processing visual and spatial information, an “episodic buffer” for combining various kinds of information into an integrated whole, and a “central executive” which controls the flow of information between components (commonly linked to sustained attention) (1, 7).

#### **2.1.2. Assessment of working memory in humans: relevance for neuropsychiatric disorders**

From a theoretical perspective, WM models have been highly influential. They have inspired the development of dozens of standardized tests to measure and research the properties and neuroscientific basis of WM. Some of earliest tests designed to assess WM processing and capacity were the “span” tests(8). These tests typically present subjects with a serial (e.g., one item per second) list of to-be-remembered stimuli. At the end of a series, the subject is required to recall or manipulate the items in the list. In the visuospatial domain, variations of these span tests emerged in later popular neuropsychological assessment tools such as the Corsi Block-Tapping Test (CBTT) (9), Self-Ordered Pointing Task (SOPT) (10), and more recent CANTAB Spatial Working Memory test (SWM) (11). The original CBTT involved repeating the actions of a researcher after they tap a sequence of identical spatially distributed blocks. The sequence

progresses from simple (e.g., 2 blocks) to more complex until the subject's performance deteriorates. This point of failure defines the Corsi Span, and averages ~5–6 for 'normal' human subjects. Task difficulty can be varied by adding delays, distractors or asking subjects to reverse the sequence order. The Self-Ordered Pointing Task (SOPT) (10) presents an array of visual items (e.g., random designs) across several different pages. Each page displays the same items, but their spatial location varies. Subjects are required to point to a different item on each page without pointing to one they have previously selected. The number of errors made across different parameters of number of items, delay and item similarity are evaluated against normative data (10, 12). The CSWM test (13) is a widely used touchscreen based paradigm that combines elements of the CBTT and SOPT. In the CSWM subjects must find a yellow 'token' hidden under one of a number of boxes. To perform efficiently, the subject must remember which items they have previously selected to avoid repetition errors. Because it is self-ordered, subjects must organize and execute an efficient search strategy. As with other paradigms, difficulty is typically progressively increased by adding boxes, delays or altering the spatial separation of the boxes.

Using these and similar paradigms, there is a wealth of data illuminating the properties of WM and its central role in cognition. Individual differences in WM capacity are associated with variation in several important abilities, including control of attention (14), non-verbal reasoning ability (15) and academic performance (16). Lower WM capacity is a central feature of a variety of neuropsychiatric disorders, including schizophrenia (17), Attention Deficit-Hyperactivity Disorder (ADHD) (18), Autism Spectrum Disorder (ASD) (19) and Major Depressive Disorder (20). It is generally acknowledged that deficits in WM are not satisfactorily treated by available pharmacotherapies (21). There is a general failure of current animal assays and models to predict drug efficacy in clinical trials, limiting their translational potential for drug discovery (22). Elucidating the neuropharmacological basis of WM and finding treatments to improve WM function are some of the most widely researched areas in cognitive neuroscience.

### **2.1.3. Assessment of working memory in rodents**

In nonhuman species, WM has been typically defined as the ability to represent a stimulus or location across a delay period and to use this information to guide subsequent actions (23, 24). Behaviors analogous to WM have been observed across a variety of phyla and species including insects, fish, birds, rodents, and nonhuman primates (25, 26) (27, 28). WM across species is commonly assessed in delayed response paradigms in which information must be remembered on a particular session or trial, but then must be flexibly replaced on later trials (29). In rodents, WM has traditionally been investigated using maze-based apparatus such as the Y, T or radial arm maze. For example, in the 8-arm radial maze, rodents are trained to collect rewards at the end of each (or a subset) of the 8 arms extending from a central circular platform (30). In a series of experiments controlling for possible perceptual (odor marking, other intramaze cues) or behavioral confounding strategies (chaining or other sequencing), the rats' performance suggested that they displayed a delay-dependent "working memory" for which arms they had previously visited within a session. Variations of other spatial navigation paradigms such as the Morris water maze, radial arm water maze and Barnes maze have also to been adapted to examine within session WM performance (29).

One of the most common delayed response paradigms is performed within a T-maze (31) or Y-maze apparatus. In a typical T-maze procedure, a rodent is initially placed in the arm at the base of the T, and allowed to traverse up the stem and freely enter one of the arms of the T. The rodent may or may not receive a reward for its choice. When the rodent begins a new trial from the base of the stem, it typically alternates or is required to alternate to earn a reward – remembering the side it had previously visited and choosing the more novel opposing side. Performance is typically degraded in a delay dependent manner.

The T-maze task is paradigmatic for a wide variety of tests with a sample phase and a subsequent 2-choice recognition test phase. For example, the object recognition test leverages a rodent's propensity to spontaneously explore novel objects. In its simplest form, a rat is presented with an object (A) or pair of identical objects in a small enclosure (sample phase). After this exposure to the objects the rodent is separated from the objects for a delay period and is then reintroduced into the enclosure with two objects: a duplicate of the previously explored object and a completely novel object. The rodents' natural tendency to explore the novel object more than the familiar object is taken as an index of recognition memory.

Although this common test is often presumed to measure short term memory due to the rapid decay in preference across increasing delays, it is difficult to separate from long term memory encoding. Variants of the classic object recognition paradigm have utilized trial unique stimuli (novel stimuli for assessing recognition memory or familiar stimuli for assessing recency) to ensure that the test is taxing short term or WM processes (32-34). These latter approaches have been far less widely implemented in the literature.

Other approaches for studying WM in rodents have shifted to operant chambers to automate and more precisely control stimulus cues and choice responses. One of the first was a delayed non-matching to position (DNMTP) task developed by Dunnett and colleagues (35). In this task, a trial begins when a rodent subject presses a lever that has been extended on one side of the chamber. During a delay period, the subject is required to make or hold its nose poke response in a central port. After the delay, two levers are presented and the subject is rewarded for pressing the non-sampled lever. While this paradigm has demonstrated that performance is delay-dependent and sensitive to lesions to the hippocampus or prefrontal cortex (36), there are concerns that subject might adopt postural or response mediating strategies to bridge the delay period, instead of using WM processes *per se*. Several studies have demonstrated such response biases (37, 38), and it remains a major concern for the DNMTP paradigm.

Most recently, rodent touchscreen-based tasks have been employed to examine visual WM processes. Visual-based automated touchscreen methods offer several general advantages compared to other classic maze-based or operant behavioral approaches (39). Like other operant tasks, experiments are automated which helps to reduce handling or interference with the animal during testing; reduce manual labor with a potential increase in throughput if multiple chambers are used to test animals in parallel; and enhance the accuracy of stimulus delivery and response recording. They are also based on non-aversive positive reinforcement (reward-based) and help minimize stress that may interfere with experimental results. Importantly, batteries of cognitive tests can be assessed using the same equipment and testing conditions, making data more easily comparable and interpretable within and across animals. There is a general failure of current animal assays and models to predict drug efficacy in clinical trials, limiting their translational potential for drug discovery (22). Quantitative assessment of cognitive performance in humans and non-human primates is increasingly tested using automated touchscreen methods (40, 41). Moreover, a battery of rodent touchscreen behavioral tasks that closely parallel human touchscreen paradigms has been developed for both mice and rats (39). Rodent paradigms that use similar

automated apparatus and paradigms may help with inter-species comparison and, thus, improve translation and back-translation.

With respect to WM tasks, a recently developed automated touchscreen paradigm is the trial-unique nonmatching-to-location (TUNL) task has been developed to help address some of the difficulties of the DNMTTP test. In the TUNL paradigm, multiple spatial locations are used in the sample and choice phases, making it more difficult for the rodent subject to predict the correct response location (39, 42). Following extensive examination during task development, it has been suggested that rats were unlikely to use a postural or other response mediating strategy. The TUNL task is believed to rely on spatial information as effective performance depends on integrity of the hippocampus. Moreover, delay-dependent impairments observed following lesions of the medial prefrontal cortex (mPFC) suggest that TUNL taps into processes akin to WM (43). While clearly progressing from prior methods, recent detailed analyses in mice suggest that mediating response biases are prevalent across numerous trial types and that further modification to task design may be required to ensure that TUNL task performance is reliant spatial WM processes (44).

Other versions of the Delayed- non-matching to sample (DNMS) task that require delayed stimulus comparison rather than simply a delayed response have been developed in operant chambers to try to minimize the possibility of mediating behaviors. Several decades ago, Pontecorvo (45) developed a continuous non-matching task using dim and bright lights as the sample and test stimuli. The position of the correct stimulus and associated lever response was randomized to prevent response bridging. While a promising approach, the task is difficult to train with considerable trial-to-trial variability and performance levels not much greater than chance. More recently there has also been development of several novel olfactory or gustatory span or nonmatch to sample WM tasks in which rodents are required to remember previously encountered odors or tastes a detect novel ones to earn rewards. While performance on these tasks is much more robust relative to tasks using visual or auditory stimuli, one major potential drawback in using odor or taste cues is that interpretation is difficult in the absence of including masking odors or tastes during the delay to ensure that there is no stimulus carryover to help bridge delays and WM load. Masking odors have rarely, if ever, been implemented in the current literature for odor and taste WM tasks.

In summary, maze-based and newer operant WM paradigms have different sets of strengths and challenges. Maze tasks typically leverage natural exploratory behaviors of rodents, but can be time and labor intensive and difficult to identify the representation used to solve the task. Operant tasks such as the DNMS or TuNL tasks can be automated with many animals run in parallel and allow more precise experimental control over stimulus presentation. However, considerable evidence suggests that animals might bridge delays with postural or movement-based mediation strategies that might significantly reduce WM load. Finally, delayed stimulus comparison tasks might help eliminate response bridging, but performance levels can be highly variable or involve stimuli (e.g., olfactory or gustatory) that are difficult to temporally control. There is a clear need for improved WM paradigms that more closely translate to human WM tasks. In this thesis we propose that the propensity for rodents to adopt mediation strategies is in part related to the availability of only two response options. We thus developed a novel Self-Ordered Working Memory (rSOWM) test modeled after the human CPTT, SOPT and CSWM tasks described above. The task enables increasing memory load through increased response options and thus potentially renders full bridging responses much less likely.

#### **2.1.4. The prefrontal cortex (PFC) in working memory.**

The prefrontal cortex (PFC) has been found to be crucial for successful execution of WM (36, 46-48). Functional brain-imaging studies of humans have identified cortical regions that are involved in spatial and non-spatial WM (49-51) with the PFC being the area involved in maintaining active representations over the delay period (52, 53). Moreover, human neurological and nonhuman primate ablation studies have amassed considerable data supporting a role for the dorsolateral PFC in delayed response and related WM tasks (54, 55).

In primates, lesion studies were among the first evidence associating WM with the PFC (46). Single unit recordings from neurons in the primate PFC later revealed that neurons persistently fire during the delay period of a delayed response task (56-59), suggesting that these neurons are somehow involved in maintaining the “task-relevant information” for a short period of time. These experiments showed that discharge from single neurons in the PFC persisted even when the physical stimuli was no longer present (named “persistent activity” or “delay activity”) (56, 59). Individual PFC neurons exhibit persistent activity in response to specific stimulus attributes, such as its location, direction of motion (60, 61), or other specific information about the stimulus that might be relevant to solve the task (62, 63). Other non-human

primate studies have shown that PFC neuronal activity decays when more than one item is held in WM (64). This is consistent with human data in which WM performance decreases as the number of items is increased. Decreased delay activity in the PFC has also been found to be predictive of errors in recall and WM performance (59, 65).

As in human and nonhuman primates, the mPFC of rats is closely implicated in efficient WM-related information acquisition (66) and retrieval (67). In the rodent touchscreen TUNL task in rats, lesion of the mPFC was found to disrupt performance in a delay-dependent manner (43). In spatial WM tasks, hippocampal-dependent allocentric coding of space is believed to be important for providing spatial information into PFC (68, 69). Such interactions are thought to be based in the prelimbic region (70, 71) owing to the existence of direct hippocampus–PFC connections (72). In a series of studies in rodents on a T-maze, there is now considerable evidence that successful task performance (alternation) is mediated jointly by mPFC and hippocampus (HPC) (73). Specifically, it was shown that the ventral HPC-mPFC input is essential for successful spatial encoding of task-related cues and their maintenance across delays. Ventral HPC-mPFC gamma synchrony is strongly correlated with successful cue encoding and was also disrupted, along with behavioral performance, by ventral HPC terminal inhibition. These findings point to a role for the ventral HPC-mPFC afferent pathway for the updating of task-relevant location information (36). Although it is somewhat controversial as to whether or how mPFC neurons might encode WM in rodents, there are numerous examples of delay-dependent activity during task performance, typically appearing transiently and sequentially during the delay period, especially during its early phase (74).

This persistent activity may be modulated by many neurochemical systems (75), including dopamine (DA) (76, 77), serotonin (5-HT) (78), noradrenaline (NA) (79), acetylcholine (ACh) (80), GABA (81) and glutamate (27, 82), which may be involved in different aspects and components of WM performance. The roles of dopamine and serotonin will be discussed further below.

### **2.1.5. PFC monoamine neurotransmitters and working memory**

Although PFC activity is controlled by many neurotransmitters, particular emphasis has been placed on the study of monoaminergic inputs to PFC – in particular DA and 5-HT – which play a crucial role in WM capacity (77, 83). A milestone in the field was the observation that DA depletion in lateral PFC induces cognitive deficits in monkeys similar to those evoked by removal of the frontal lobes (47).

### 2.1.5.1. Dopamine

The neurotransmitter DA is synthesized by dedicated neurons located in the midbrain. Midbrain DA neurons in the ventral tegmental area (VTA) project to PFC (e.g., anterior cingulate cortex and medial prefrontal regions) via the mesocortical dopamine pathway. Primates have additional projections from dorsolateral and lateral substantia nigra (SN) to granular dorsal and lateral areas of the PFC. When dopamine neurons fire, dopamine is broadly released from varicosities and terminal endings and is generally thought to act as a neuromodulator to enhance or suppress synaptic transmission indirectly.

Once dopamine is released it can bind to five different dopamine receptor subtypes, D1–D5. These receptors are G protein-coupled receptors (GPCRs) that modulate intracellular signaling cascades rather than producing postsynaptic currents directly (84, 85). Dopamine receptors fall into two main families: the dopamine D1 receptor family (D1R) with subtypes D1 and D5, and the dopamine D2 receptor family (D2R) with subtypes D2, D3, and D4. In primates, D1Rs are expressed in all cortical layers in primate PFC and are about 10-fold more abundant than D2Rs (86). In rodents, D1 is also more abundant than D2 in mPFC, with the highest density found in deeper cortical layers V and VI. On the other hand, D2Rs have generally low expression rates, with slightly higher expression in layer V for primates (86, 87) and more superficial layers of mPFC in rodents. D1Rs and D2Rs are expressed in both pyramidal cells and inhibitory interneurons (88–90). The ultimate effects of dopamine release in PFC are likely to vary depending on receptor subtype, cell type, current synaptic properties, and on interactions with other transmitters (85).

Unlike the wealth of data in the striatum, dopamine transients in mPFC of rodents do not appear to directly mediate reinforcement learning but may instead signal the presence of behaviorally relevant stimuli (91, 92). It is generally believed that dopamine acts on different timescales to influence processing in consecutive stages of complex working memory tasks, such as delayed response tasks, impacting on distinct populations of PFC neurons to highlight a stimulus, maintain relevant information in working memory and prepare to execute a goal-directed action.

Dopamine neurons projecting to PFC generally appear to be active when the animals successfully detect a stimulus in a delayed reward paradigm (93). The precise timing of the dopamine neuron responses reflected the subsequent choice of the animal and closely aligned with the latency of neuronal choice signals in frontal cortex; but were slightly delayed offset relative to signals in visual cortex (94). This

suggested that dopamine transients in PFC may provide a gating signal that allows input to become updated and stored in working memory (95-97).

Parallel electrophysiological recordings from sensory areas and dopamine neurons suggest that their timing converges in PFC. Based on this and related evidence it is generally believed that dopamine may directly modulate sensory input to PFC and act as a gate for behaviorally relevant information to be encoded in prefrontal networks (84). In this gating mechanism it is hypothesized that D1Rs first increase neuronal signal-to-noise ratio via inhibitory mechanisms. D2Rs are then thought to mediate a secondary gain computation to enhance sensory coding by excitatory mechanisms. This gating hypothesis depends on the as yet unobserved onset of phasic dopamine signals in PFC are temporally precise, as suggested by dopamine neuron activity (96, 98-101). This study will be one of the first to examine the dynamics of these phasic dopamine signals in PFC during working memory task performance. The main argument against a gating hypothesis is the reported slow and long-lasting dopamine levels in PFC (102).

In addition to enhancing sensory processing of behaviorally relevant information, numerous behavioral studies have established that prefrontal dopamine alters working-memory performance in both humans and animals (85, 102, 103) for review).

As described above, it is thought that neurons use sustained activity to actively buffer and process information so as to bridge the temporal gap until an adaptive output response is selected (104-106). Such persistent activity in the absence of external cues or input is typically modeled by recurrent connections within the active population of PFC neurons, such that excitation repeatedly re-activates neuronal circuits via recurrent feedback loops.

Systemically blockade of D1Rs impairs spatial working memory in the ODR task (107, 108), whereas stimulation of D1Rs has been found to improve working memory performance specifically in dopamine-depleted animals (109). The effects of D1R activation are dose-dependent and follow an inverted-U response curve, where too little or too much D1R activation is detrimental for working memory performance (110, 111).

While considerable evidence exists implicating a role for dopamine in modulating working memory or other executive task performance on relatively broad timescales (e.g., tens of seconds to minutes or longer), due to technological limitations, comparatively little work has been done to examine

the phasic dynamics of endogenous dopamine directly in mPFC during working memory task performance. In chapters 5.2 and 5.3 of this thesis, I will investigate the real time phasic dynamics of both dopamine and serotonin during stimulus presentation, choice response execution, delay encoding and waiting, and reward collection within a working memory (rSOWM) task and in tandem with a delay discounting (rDRD) task.

### **2.1.5.2. Serotonin**

Much less is known about the specific role of serotonin (5-HT) in working memory and other cognitive functions (112-114). Serotonin is produced in a small cluster of neurons located in the brainstem raphe nuclei that project extensively to the whole neuroaxis. These nuclei can be subdivided into caudal and rostral portions, where the rostral group provides the main 5-HT projects to the central nervous system (CNS). The dorsal raphe (DRN), median raphe and suprallemniscal nuclei of the rostral group all project to the PFC, with distinct populations of cells projecting to different subregions of prefrontal cortex (including mPFC, anterior cingulate cortex and orbitofrontal cortex). Although the median raphe nucleus projects to mPFC, the strongest number of 5-HT projections is from the DRN to mPFC.

The 5-HT projections to mPFC are relatively dense and span all layers. As for the dopaminergic system, most cortical serotonergic fibers lack specializations and traditional synapses (only ~25% in rodents and nonhuman primates), and thus 5-HT signaling with the mPFC is believed to occur through volume transmission. The greatest incidence of synapses are reported in the superficial layers of the prelimbic mPFC (115-117). Based on 5-HT transporter levels and distribution, released 5-HT is believed to diffuse relatively widely.

At least 15 serotonin receptors have been cloned and identified, which can be grouped into seven families based on their signaling mechanisms (118). Thus 5-HT modulation of mPFC function is heterogenous based on local subregion, receptor subtypes present and where they are located. 5-HT receptors on cell bodies and dendrites of mPFC neurons appear to differentially regulate specific output circuits (119). There is also heterosynaptic 5-HT regulation of both proximal and distal axon terminals of mPFC afferents as well as of mPFC neurons themselves. The PFC has abundant expression of 5-HT<sub>1A</sub> and 5-HT<sub>2A</sub> receptors (120, 121) where they are believed to exert opposing functional effects (5-HT<sub>1A</sub> receptors = inhibitory; 5-HT<sub>2A</sub> receptors = excitatory). Both receptors are expressed by pyramidal neurons

and by GABAergic interneurons, adding a further element of complexity. In the rat, more than half of prefrontal pyramidal neurons express 5-HT<sub>1A</sub> or 5-HT<sub>2A</sub> receptors (121-124). Despite their presumed opposing functional effects, the majority of these pyramidal neurons co-express both receptors (125-127). Their differential distribution on neuronal compartments may help explain: 5-HT<sub>1A</sub> receptors are primarily expressed on the axon initial segment of pyramidal neurons where they act to suppress action potential generation (122, 128, 129), whereas 5-HT<sub>2A</sub> receptors are most abundant in apical dendrites (78, 122), where they may amplify the impact of excitatory synaptic currents. Overall, the serotonergic regulation of prefrontal cortex is diverse and correspondingly is implicated in diverse circuits and in modulating top-down control of other brain regions.

While 5-HT has been less investigated than dopamine in the context of working memory research, several studies have investigated the role of 5-HT in modulating dopaminergic neurotransmission within the prefrontal cortex. This interaction is believed to be mutually opposing. 5-HT displays functional tonic inhibitory control over DA, as lesioning the MRN or DRN increases the metabolism of DA in the nucleus accumbens (130). Moreover, 5-HT<sub>2C</sub> receptors in general tonically inhibit DA release. Blocking 5-HT<sub>2A</sub> receptors has been indirectly linked to increases in dopamine release in the prefrontal cortex (131, 132).

Indeed, one of the more prominent theories of dopamine and serotonin function is that their opponency might mediate the coding of the full spectrum of affective valence. While there is evidence to support opposing roles of DA and 5-HT on tonic firing and neurotransmitter levels, it has only been speculated that a phasic serotonin signal might report an ongoing prediction error for future punishments to complete the model (130). They note that “a key requirement here is a spatially and temporally far finer scale view of the activity of 5-HT (and DA) neurons and release in target structures” (133).

In chapters 4.2 and 4.3 of this thesis, by expressing novel GRAB sensors for 5-HT (and DA) in the mPFC of mice, I will directly examine the dynamics of phasic 5-HT and DA release during performance of a working memory task (rSOWM) as well as delay discounting task (rDRD) which also involves choice, delays and implicates a role for prefrontal 5-HT and DA.

## **2.2. Delay Discounting**

### **2.2.1. Delay Discounting definition and psychopathology**

Delay discounting refers to the phenomenon whereby delayed outcomes, such as delayed rewards, are valued less than when those outcomes are available immediately or with a shorter delay (134). This tendency to devalue delayed outcomes occurs across species and is often well described by exponential or hyperbolic discounting curves with outcome value decaying as a function of increasing delay.

### **2.2.2. Assessment of delay discounting in humans**

Human discounting tasks typically use questionnaires or surveys to examine choice behavior (e.g., A series of questions of the type: “Would you rather have \$10 now or \$50 in one month?”). For ease of data collection and analysis, researchers often only systematically alter the delay of the large reward for a given small reward to estimate multiple indifference points, which are theorized to represent the discounted value of the reward (135). An alternative method is to adjust the amount of reward for a set of fixed delays (136). The fitted slope of the obtained indifference points is commonly designated as the discounting rate,  $k$ , and is generally found to be stable across the different methods (137). Recent variants in human discounting have also devised tests to estimate discounting rates without the use of indifference points (138, 139). Recently, there has been growing interest in discounting tasks that use actual (primary) rewards and delays, rather than the hypothetical ones described above, to more closely map to real world scenarios and to better translate to studies in animals.

Several psychopathologies are characterized by maladaptive or steeper levels of delay discounting, including substance use disorder, pathological gambling and attention deficit hyperactivity disorder. While delay discounting tasks are traditionally viewed from the lens of impulsivity, behavioral inhibition and dysregulated choice, there is growing recognition of strong links with working memory. Studies have demonstrated that working memory training can help to reduce delay discounting rates. For example, a study found that adults in treatment for addiction to stimulants showed significantly reduced DD rates after receiving working memory training. Moreover, several studies have reported that discounting rates are inversely correlated with working memory performance and that increasing working memory load can lead to increased discounting rates.

### 2.2.3. Assessment of delay discounting in rodents

In rodents, there are three procedures most commonly used to measure delay discounting: the fixed-delays schedule; the adjusting-delays schedule and the adjusting-amounts schedule. An operant, discrete-trial, fixed-delays procedure was pioneered by Evenden and colleagues (140). Briefly, subjects are trained to initiate trials to extend two levers into the chamber (Figure 1). One lever yields immediate delivery of a small reward while the other lever yields a larger reward. A delay prior to receiving the large reward after lever press is increased progressively across blocks of trials. Although the indifference point is not directly estimated, it may be inferred from where the discounting curve crosses 50% preference. The adjusting-delay schedule was originally devised by Mazur (136). In this procedure, subjects perform a series of trials in which the delay to receive a smaller reward is fixed and the delay to a larger reinforcer varies according to the subject's choices. Choice of the larger reward increases the delay to receive the large reward on the next trial. If the smaller reward is chosen, the delay to the large reward is reduced on the next trial. The adjusting-amount schedule (141) is similar to the adjusting rewards schedule except that the reward magnitude is adjusted while the delays are kept constant.

Relative to the fixed delay procedure, the adjusting procedures offer more comprehensive quantitative estimation of subject's preferences and enable a subject's sensitivity to reinforcer delay to be distinguished from sensitivity to reinforcer magnitude (142). However, the training can be extremely lengthy for the adjusting procedures and there is little opportunity to study acute manipulations, such as pharmacological interventions. Moreover, sensitivity to the delay was not readily observed on a trial-by-trial basis, at least for the adjusting delay procedure (143). Moreover, the standard protocols for both fixed and adjusting procedures designate a fixed location (e.g., lever position) for the response options, which may lead to positional biases when responding in the paradigm. This configuration makes it difficult to separate either sensitivity to differences in reward amount or side bias from actual choice preference based on value. While the adjusting procedures have a can fit a bias parameter for 0s delay trials (144), this is an average measure and does not account well for local runs or "stickiness" behavior.

To address these putative limitations and to further quantitatively assess discounting to both delay and reward magnitude, we developed a novel touchscreen delay and reward discounting paradigm (rDRD). Rodents are trained to respond to three main 2-choice trial types: trials when delays are equal but magnitudes are different (20%); trials when magnitudes are equal but delays are different (20%); and trials in which there is a smaller sooner versus a larger later reward (60%). Stimulus options are presented randomly across response windows and forced choice are programmed (with >3 choices in a row on the

same side) to help prevent development of side biases. Moreover, the multiplex delay/reward magnitude combinations are well aligned for analysis with multilevel logistic mixed effects regression modeling for extracting separable parameters for sensitivity to delay and reward magnitude at the level of the population and of individual subjects.

#### **2.2.4. PFC and neurotransmitters in delay discounting**

Unlike delayed response tasks or other paradigms that tax executive control in making decisions to earn rewards and avoid mistakes, delay discounting is instead designed to assess preference between two alternatives varying in delay and/or reward magnitude. Thus, different circuitries are differentially engaged in the two classes of paradigms. The orbitofrontal cortex (OFC) and nucleus accumbens (NAc) are two of the main brain regions that are engaged and thought to mediate task performance. Both of these brain areas are commonly reported to encode reward value. Lesions to the OFC or NAc in nonhuman primates and rodents have typically been observed to result in increased discounting rates (impulsive choice) (145, 146) (147, 148).

While disruption of mPFC remarkably does not greatly impact delay discounting behavior (147), some evidence does suggest that dopaminergic (DA) and serotonergic (5-HT) activity within mPFC may contribute to choice behavior. Elevations of intra-mPFC 5-HT efflux have been observed in animals performing a progressive delay discounting procedure (149). Given the critical role for DRN activity and 5-HT signaling during tasks involving waiting for rewards (150, 151), these elevated 5-HT levels may reflect integrating across numerous waiting periods. For dopamine, decreasing DA levels in mPFC increases an animal's preference for a small, immediate reward (152, 153) in a fixed delay procedure. Similarly, selective D2 receptor antagonists quinpirole (1.25  $\mu$ g) or eticlopride infused into mPFC was also observed to increase impulsive choice behavior (154). In contrast to the relative sparse effects observed in mPFC, there is a wealth of data showing that depletion of DA or 5-HT or infusion of DA or 5-HT receptor antagonists into the OFC can severely disrupt delay discounting choice behaviors.

In summary, to date, little is known regarding the influence of the monoaminergic neurotransmitters - dopamine and serotonin - in the mPFC. Although it is clear that working memory tasks and delay discounting tasks both require animals to successfully bridge delays to optimize performance and rewards, the differing task demands appear to preferentially recruit distinct prefrontal subregions and circuitries. However, phasic signals for DA and 5-HT have never been recorded in the mPFC during touchscreen delay discounting (rDRD) performance. We predict that there will be both common and

distinct release dynamics in comparison to working memory (rSOWM) task performance that will help clarify and interpret the main findings.

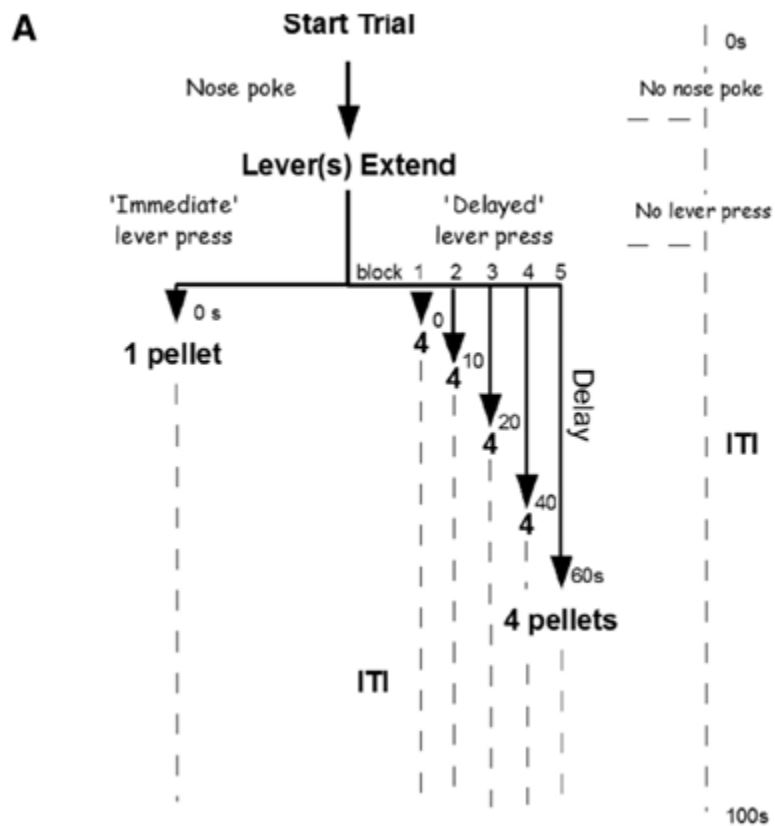


**Figure 1.** Outline of the delay-discounting task used for rats. From Mar, AC. et al., 2007 (146).

## 2.3. Optical imaging of prefrontal DA and 5-HT dynamics

### 2.3.1. Fiber photometry overview

Monoamine neurotransmitter dynamics in the PFC of rodents have largely been investigated using techniques such as microdialysis that have relatively slow temporal resolutions. The emergence of *in vivo* optical techniques such as fiber photometry, as well as the recent development of genetically encoded fluorescence-based biosensors, has introduced new technological means to measure neurotransmission in awake animals.

Fiber photometry is increasingly used as the state-of-the-art technique to measure cellular and neurotransmitter dynamics *in vivo* in rodents (155). It was originally designed to measure intracellular calcium fluctuations using genetically encoded indicators and serve as a sensitive and real time proxy for neuronal activity. The flexibility and specificity of genetic encoding has enabled interrogation of defined cell types and circuits underlying sensory processing, motor behaviors, memory and motivation. Very recently, fluorescent sensors for biomolecules, including receptors for neuromodulators and peptides that were previously inaccessible *in vivo*, have been developed. Along with the more widespread availability and affordability of commercial optical recording systems, this technical breakthrough has now made it highly accessible to monitor molecules with high spatiotemporal precision during behavior.

Fiber photometry is an optical technique in which light is used to trigger and measure fluctuations in fluorescence that arise from conformational change of an expressed biosensor. Excitation light of a specific wavelength can be delivered through an implanted optical fiber, and emitted fluorescence from an expressed fluorophore can be routed to a highly sensitive photodetector along the same fiber. A digital optical signal (e.g., voltage) is generated by the photodetector that is thought to indicate the relative amount of regional (bulk) activated sensor in close proximity (50 to 400  $\mu\text{m}$ ) to the tip of the fiber. As biosensors are genetically encoded, their expression can be directed to defined circuits and/or cell types and they can remain stably expressed for several weeks to months.

Fiber photometry has a higher spatial resolution and much higher temporal resolution than typical microdialysis experiments that have sample rates of many seconds to minutes. This was clearly demonstrated by within-subject recordings of dopamine using GRAB sensor photometry in parallel with standard microdialysis (which missed all the phasic dynamics). Moreover, compared with cyclic

voltammetry, photometry can offer greater sensitivity for some analytes/environments. For example, measuring dopamine in vivo with chronically implanted carbon-fiber microelectrodes using fast scan cyclic voltammetry (FSCV) is challenging, particularly in regions such as prefrontal cortex where dopamine levels are low relative to other monoamines such as norepinephrine. From a practical standpoint, fiber photometry involves relatively straightforward, noninvasive stereotaxic surgical procedures and use of lightweight optical fibers to record signals are less restrictive to natural behavior than many other techniques.

In summary, fiber photometry enables researchers to sensitively monitor fluctuations in biomolecules or physiological events across multiple timescales, from sub-second to several hours. This is crucial for the types of in vivo experiments performed in this thesis, where the fundamental mechanisms of information encoding and computation are not yet known.

Comparison of typical features of *in vivo* detection methods

|                                             | Fiber photometry         | E-Phys               | FSCV                    | Microdialysis         | Mini-scope | Two-photon scope |
|---------------------------------------------|--------------------------|----------------------|-------------------------|-----------------------|------------|------------------|
| Cell specificity                            | yes                      | limited <sup>a</sup> | no                      | no                    | yes        | yes              |
| Molecular specificity                       | yes                      | no                   | yes <sup>b</sup>        | yes                   | yes        | yes              |
| Spatial resolution (cell or subcellular)    | multiple cells/processes | cellular             | regional (~100 $\mu$ m) | regional (~1 mm)      | cellular   | subcellular      |
| Temporal resolution                         | 10–100 ms <sup>c</sup>   | <1 ms                | 100 ms                  | 10 min                | 10 ms      | 10 ms            |
| Setup cost (thousands of US\$) <sup>d</sup> | \$5–\$25                 | \$5–\$100            | \$10–\$30               | \$8–\$65 <sup>e</sup> | \$5–\$250  | \$125–\$300      |

The values provided are in the typical practical range used and do not represent theoretical limits of the techniques.

**Table 1. Practical comparison of in vivo detection methods. Table from Simpson EH, et al., 2024(155).**

Although classic detection methods such as microdialysis (156) and fast-scan cyclic voltammetry provide valuable accesses for measuring 5-HT levels, they lack the high spatiotemporal resolution, specificity and sensitivity and minimal invasiveness needed for the in vivo detection of 5-HT and DA.

### 2.3.2. GRAB sensors

One of the major goals of this thesis is to characterize the detailed phasic dynamics of dopaminergic and serotonergic transmission in the medial prefrontal cortex in real time while animals are engaged in tests or working memory or delay discounting. To accomplish this goal we employed recently developed genetically encoded optical tools enabling us to monitor the concentration and the dynamics of dopamine and serotonin in the extracellular space of the prelimbic region of prefrontal cortex with high spatiotemporal precision. These tools comprise a set of green and red fluorescent G-protein-coupled receptor (GPCR) activation-based (GRAB) sensors based on GPCR receptors which are selective for dopamine or serotonin. The GRAB sensors for DA and 5-HT are composed of two main components: a ligand binding module (modified receptor subtype) that binds the DA or 5-HT molecule, and a fluorescent module containing a fluorescent protein. The ligand binding domains are dopamine receptor D1 (D1R) or D2 (D2R) based GRAB DA for dopamine and serotonin receptor 4 (5-HT4R) GRAB 5-HT for serotonin; these domains are combined with conformation sensitive, circularly permuted fluorescent proteins 74,75. Upon binding of DA or 5-HT, the conformational change of the ligand binding module leads to alterations in the signal produced by the fluorescent module (within 10s of milliseconds). These GRAB sensors usually show a relatively high signal to noise ratio (SNR) and are highly suitable for in vivo recordings.

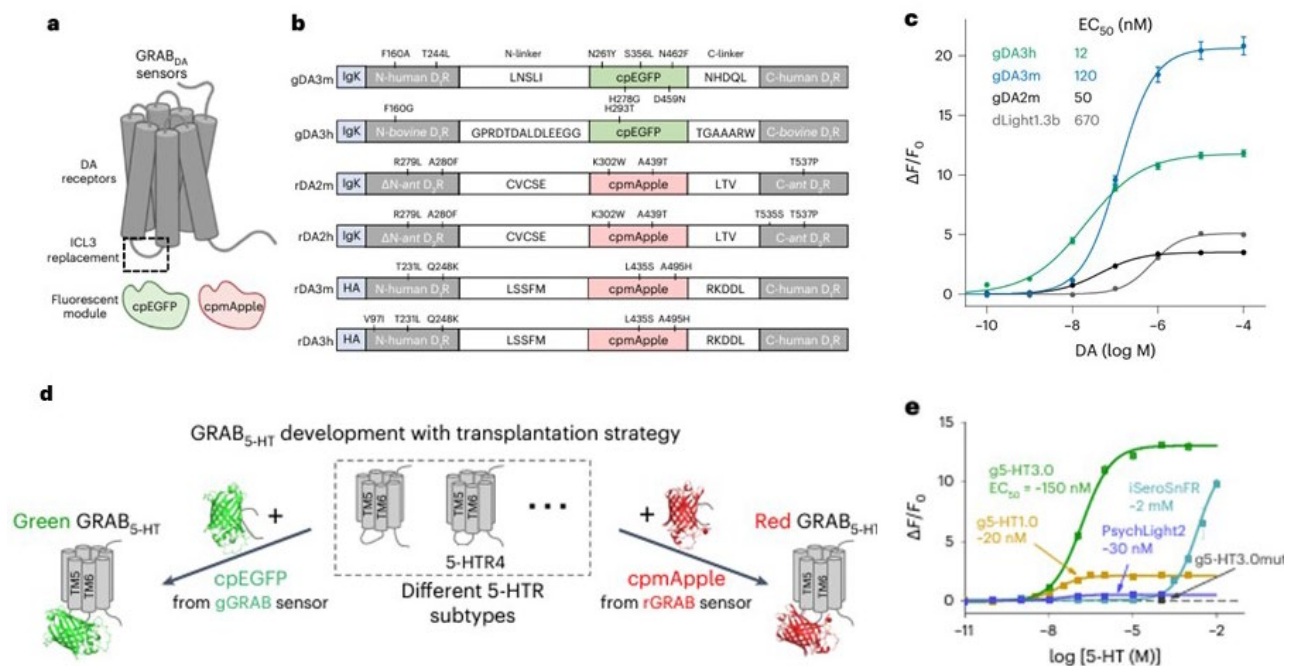


**Figure 2. Development of improved dual-color (green and red) DA and 5-HT sensors.** **a)** Schematic illustration showing the principle of next-generation DA sensors. **b)** Schematics of improved GRAB<sub>DA</sub> sensors. Mutations are indicated with respect to wild-type receptors and fluorescent proteins. Immunoglobulin  $\kappa$ -chain (Igk) leader sequence or histamine (HA), N terminus leader sequence. **c)** Spectral profiles of GRAB<sub>DA</sub> sensors. One-photon excitation (top-left), emission (top-right) and two-photon excitation (bottom) spectra of indicated sensors with (dashed lines) or without (continuous lines) DA are shown. **d)** Top, the strategy for developing GRAB<sub>5-HT</sub> sensors. Bottom, performance of sensor candidates based on different receptor subtypes for green (left) and red 5-HT sensors (right). The dashed horizontal line represents g5-HT1.0 response (bottom left), and the best candidate green and red sensors are denoted by enlarged green and red dots, respectively. **e)** Dose–response curves of different 5-HT sensors.  $n = 3$  wells for each sensor, with 300–500 cells per well. **Images and legend adapted from Zhuo Y., et al. 2023 (a-c) Deng F., et al. 2024 (d,e).**

The development of these sensors is advancing rapidly and new tools with greater signal and sensitivity are quick to replace older generations of sensors. Indeed, over the course of this thesis, one of the challenges of previous sensor versions was their limited sensitivity and resolution in DA detection in sparsely innervated regions such as the mPFC. The latest generation of these sensors (as detailed in Methods) proved to be more sensitive and able to capture the dynamics and transients of DA and 5-HT changes during cognitive testing. We leveraged these sensors to obtain the first high temporal resolution fiber photometry recordings of DA and 5-HT fluctuations during working memory task (rSOWM) or delay discounting task (rDRD) performance.

### 3. Hypothesis and objectives

In this thesis I have several stepwise and interrelated hypotheses:

#### **Rodent Self-Ordered Working Memory (rSOWM) paradigm**

- 1) Increasing the number of response options in the novel rSOWM task in rats will increase working memory load and render it more sensitive for detecting cognitive impairments or enhancements
  - a. Dentate gyrus lesions will result in deficits in pattern separation (inter-stimulus distance) while damage to CA3 will cause impairments under conditions of increasing stimulus number or delay in the rSOWM task.
  - b. Systemic modafinil administration will enhance rSOWM performance particularly under conditions of increasing stimulus number or delay, mirroring the nootropic effects of modafinil in the human CANTAB SWM task.
  - c. Microinfusion of modafinil directly into the medial prefrontal cortex will augment rSOWM performance, mediated at least in part by enhancement of the dopaminergic system.
- 2) The novel rSOWM task will measure working memory in mice, with task performance sensitive to memory load and delay
  - a. Medial prefrontal dopamine release fluctuations will track key task events (e.g., stimuli, actions, rewards), and be associated with rSOWM performance at higher WM load.
  - b. Medial prefrontal serotonin release fluctuations will track key task events (e.g., stimuli, actions, rewards), and be elevated by waiting during delays prior to stimulus presentation.

#### **Rodent Delay and Reward magnitude Discounting (rDRD) paradigm**

- 3) The novel rDRD task will quantitatively measure individual differences in delay discounting and reward magnitude sensitivity
  - a. Medial prefrontal dopamine release fluctuations will track key task events (e.g., stimuli, actions, rewards), and will not be associated with waiting during delays prior to reward delivery
  - b. Medial prefrontal serotonin release fluctuations will track key task events (e.g., stimuli, actions, rewards), and be elevated by waiting during delays prior to reward presentation.

This project was divided into three main aims:

**Aim 1.** To develop a novel, automated translational paradigm (rSOWM) to study WM in rats that is sensitive to parametric manipulations (e.g., stimulus number, delay or spacing) known to impact WM performance.

- a) To validate the rSOWM task with neural and pharmacological manipulations.
- b) To assess WM task sensitivity to hippocampal (e.g., CA3 and dentate gyrus) lesions.
- c) To assess WM task sensitivity to putative cognitive enhancers (Modafinil, mgluR5 PAM) and investigate the involvement of the mPFC if effects are observed.
- d) To investigate whether modafinil can improve rSOWM performance via local infusion into the prefrontal cortex.

**Aim 2.** To adapt and further validate the task in mice.

- a) To examine the real-time temporal dynamics of prefrontal DA and 5-HT on rSOWM performance. I did this using recently developed GPCR-based GRAB DA and 5-HT sensors in conjunction with fiber photometry methods. DA and 5-HT release changes are measured at a high temporal-resolution and aligned to the main rSOWM trial events: delay, choice, reward collection.

**Aim 3.** To develop and validate a novel parametric and quantitative Delay and Reward Discounting (DRD) task for rodents to better understand the role of PFC monoamines during adaptive choice based on reward and delay processing (discounting) that is independent of WM. This includes examining whether choice performance is sensitive to both delay and reward magnitude and apply a logistic regression model to better understand the role of each component (also existing in rSOWM).

- a) To study the role of prefrontal DA and 5-HT on rDRD performance using fiber photometry. As the delay phase in rDRD occurs after the choice is made and is WM independent, comparison of DA and 5-HT release dynamics during delay, choice and reward collection phases in rDRD and rSOWM is key to better understand their role in WM and other delayed reward paradigms.

## 4. Material and methods

### 4.1. Animals

A total of 72 adult Lister hooded rats and 115 C57BL6J mice were used in these projects. Rats were obtained from Charles River (UK), weighing 225-275 grams at arrival. Rats were housed 3-4 per cage and maintained on a reverse day/night light cycle (lights on 7pm–7am) and tested during the dark phase. Once training began, rat chow (Purina) was restricted to maintain animals at 85-90% of normal body weight. Rats were habituated to the facility and handled for one week prior to behavioral training. Three cohorts of adult (>4 months) male adult rats were used in this study. Cohort 1 (n=31) were for the hippocampal lesion experiment (Chapter 1). Cohorts 1 (n=22) and 2 (n=19) were used for rSOWM task validation and systemic and central pharmacology. All experimental procedures on rats were conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Three-month old female and male mice were obtained from Jackson Laboratories. Mice were housed 3-5 per cage and maintained on a reverse day/night light cycle (lights on 10pm–10am) and tested during the dark phase. Animals were acclimated to the housing rooms for at least two weeks after receipt and handled for a week prior to behavioral training. Food was restricted to maintain animals at ~90% of normal body weight. Five separate cohorts of male and female adult mice were used for this study: 51 (26 males and 25 females) were used for all the rSOWM experiments (Chapter 2), and 64 (32 males and 32 females) were used for all the rDRD experiments (Chapter 3). All experimental procedures were conducted in accordance with the NYU Langone Health (NYULH) Institutional Animal Care and Use Committee (protocol no. IA16-02082) and in compliance with the Guide for Animal Care and Use of Laboratory Animals.

## 4.2. Behavioral testing apparatus

Both the rSOWM and rDRD tasks were developed to be used in standardized automated touchscreen chambers (Med Associates or Campden Instruments)<sup>30</sup> (**Figure 3**).

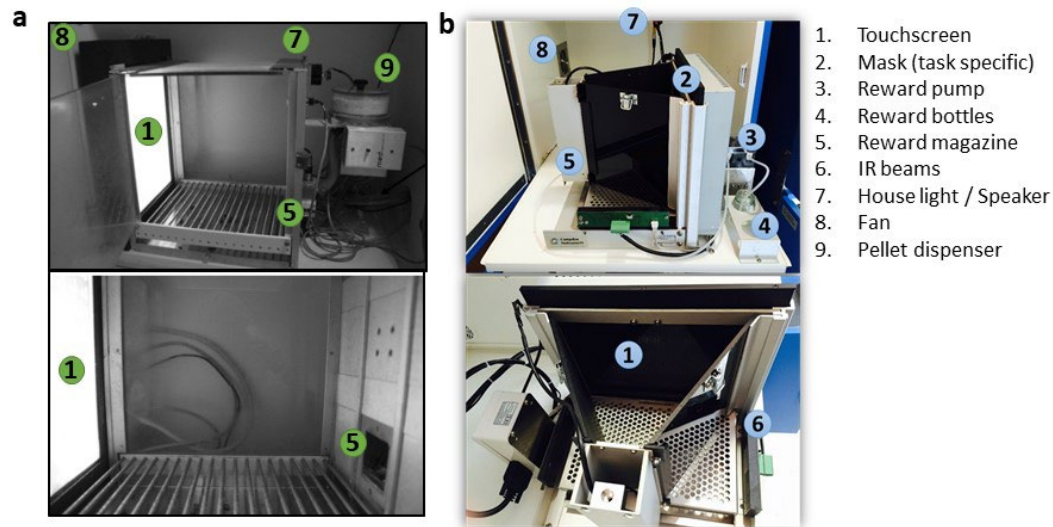


**Figure 3.** Rat and mouse operant boxes. **a)** Med Associates (Vermont, USA) rat touchscreen chambers. The inner chamber is 25cm high, 25cm deep, and 30cm wide consisting of a metal frame with clear Perspex walls. The floor consisted of stainless-steel bars spaced 1cm apart and 3cm above a waste tray. The touchscreen monitors (23cm x 29cm viewable area, Craft Data Ltd., Chesham, UK), at one end of the chamber, register touch by infrared detectors interfaced by ELO touchscreen software (ELO Touchsystems Inc). On the wall opposite from the monitor was a food magazine equipped with a 3W light, an infrared detector to register nose pokes (Med Associates Inc., Vermont, USA) and a pellet dispenser (Med Assoc. ENV-203-45) delivering 45mg 5-TUL AIN-76A dustless pellets (TestDiet, Indiana, USA). Above the food magazine was a house light (3W), and tone generator (Med Assoc. ENV-223HAM). Each operant box was housed within a sound-attenuating chamber equipped with a small fan. The boxes and monitors were controlled using IBM Netvista computers running custom programs written in Microsoft Visual Basic .NET 2010. **b)** Campden Instruments model touchscreen chamber package (#80604-20 for rats, #80614-20 for mice) equipped with: screen, tone generator, house light, food magazine, reward dispenser, front and rear infrared beams, and sound attenuation cubicle. The reward dispenser for rats (80209-45) delivered 5-TUL AIN-76A dustless pellets and the dispenser for mice comprised a peristaltic pump (80204A) that delivered 10% sweetened condensed milk (La Lechera, Nestle). The inner chamber is trapezoidal in order to focus the animal's attention on the touchscreen and reward delivery area. The inner chamber has black Perspex walls 30cm high, 30cm long, 25cm wide at screen and 18cm wide at pellet dispenser. The floor is a grid 2cm above a waste tray. The magazine is equipped with a light and a sensor to detect nose-pokes. The chamber is also equipped with a house light that is used as a negative feedback after an

incorrect response. An IR camera above the chamber points through the transparent lid, enabling monitoring of animal behavior on computers outside the testing room to minimize interference during the testing. The boxes are controlled in groups of four via a model 88520 Four Station Touch CPU. The boxes are controlled in groups of four via a model 88520 Four Station Touch CPU. Customized programs were written in Microsoft Visual Basic .NET 2010 or ABET II VideoTouch v21.02.26.0 and run using Whisker Server (Version 3.6.1, Cardinal and Aitken 2001).

### **4.3. Rodent Self-Ordered Working Memory (rSOWM) task**

#### **4.3.1. rSOWM task design**

The rSOWM task was developed to include elements of the self-ordered pointing task (SOPT; (10) and the Cantab Spatial Working Memory test (Cambridge Cognition) widely used to assess working memory in human psychometrics. In the final rSOWM task, rats or mice were required to nose-poke into the food receptacle to start the session and, subsequently, each trial. This triggered presentation of 2, 3 or 4 white square stimuli on the screen. The number, horizontal location and distance between stimuli were randomly selected on each trial. When a square was touched, all stimuli were immediately removed from the screen. A response to an unvisited square (“correct response”) triggered a tone paired with the delivery of a reward (one 45mg-sugar pellet for rats and 20μl of 10% sweetened condensed milk for mice) and activation of the food receptacle light. After reward collection, the same stimuli were presented to continue the trial. After all stimuli had been selected once on a given trial, the trial was deemed complete and was followed by an inter-trial interval (ITI) period (20-40s) before the next trial began (Figure 4a). A square revisit (“incorrect response”) triggered the presentation of a visual stimulus (‘fish skeleton’) in the incorrect location and the activation of the house light for 5s before the stimuli reappeared (Figure 4b). To increase task difficulty and in some sessions delays were introduced in which a 3 or 6s waiting period was added before the stimuli reappeared after each response. Delays were added pseudo-randomly to half of the trials within the session. Animals were given 1-2 training sessions of 60-90 min (or 100 trials maximum) daily.

Task performance was assessed using the percentage of perfect trials ( $\% \text{perfect} = \text{perfect trials} / \text{total trials}$ ), in which each stimuli option was selected only once; and the proportion of errors in non-perfect trials ( $\text{NPerrors} = \text{errors} / (\text{total trials} - \text{perfect trials})$ ) or revisits to previously-selected stimuli. Reaction times and latencies to respond and collect rewards were also measured. Animals had full control over the order or sequence of touches. Probabilities to use each possible sequence of responses as well

as the entropy of their choices were calculated to analyze the sequencing patterns across sessions and the different trial types. Entropy was calculated as follows:

$$H(X) = - \sum_{i=1}^n P(x_i) \log P(x_i) \quad \text{where } P \text{ is the probability of each sequence order used by the animals.}$$

An experimenter-ordered variant of rSOWM, we named rEOWM, was designed to probe whether performance is impacted when animals are allowed to strategically order their choices (see Results Figure 9). This variant was similar to the main procedure with the exception that, only one stimulus was displayed at the beginning of the trial. After the first stimulus is chosen the trial proceeds as normal rSOWM. The starting position for the first stimulus was randomized and balanced across all relevant parameters. The experimentally ordered task variant is particularly useful for detecting certain behavioral strategies and position biases.

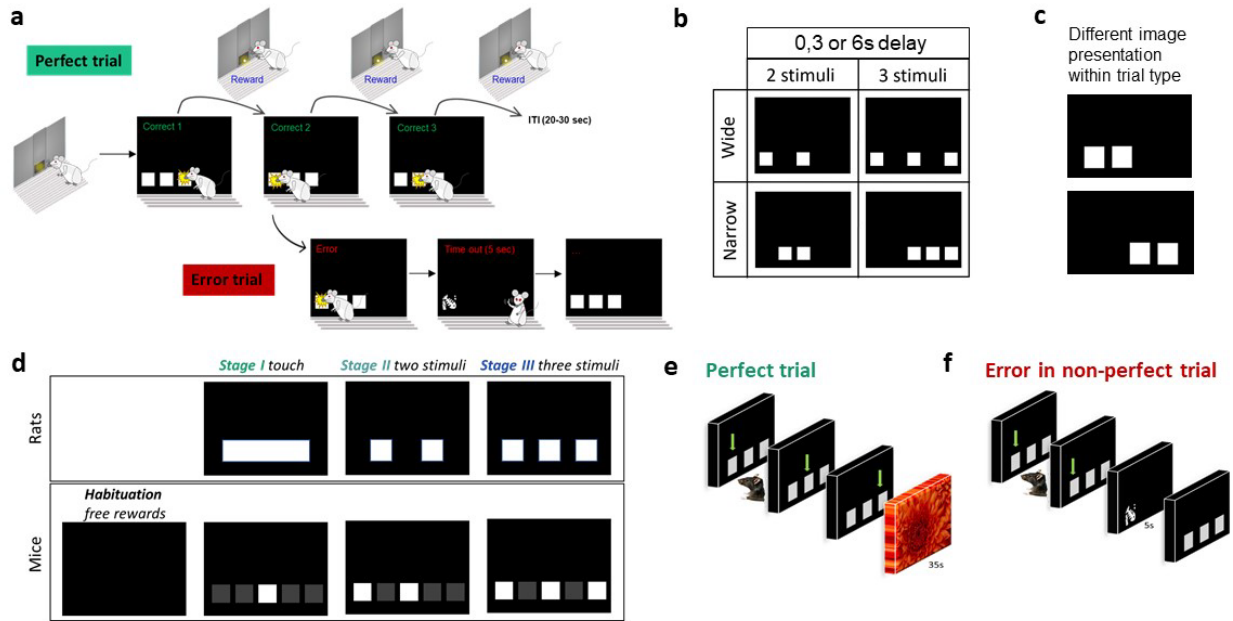


**Figure 4.** Schematic representation of the rSOWM behavioral paradigm. **a)** Representation of the different trial types used in rSOWM with different delays, stimulus number and separation. The stimuli consisted in white squares (4.35x4.35cm) located in the same row, 4.35cm above the floor. Varying locations and distances between stimuli were used on each trial within a session. Separations were wide (4.35cm) or narrow (2.18cm). **b)** Trial type representations by number of stimuli (2 or 3), separation between the stimuli (narrow and wide) and delay (0, 3 or 6s). **d)** Training stages for rats and mice were given to the subjects prior to the full rSOWM task version (a). **c)**

Representation of two different trials with the same trial characteristics but presented in different screen locations. e) Schematic of a perfect trial. f) Schematic of an incorrect response in a non-perfect trial.

#### **4.3.2. rSOWM training protocol for rats**

Animals learned the basic operation of touchscreen chamber and the task structure through a series of training stages. Each animal typically received one daily training session (5-6 sessions/week). Animals were food deprived according to national and institutional guidelines (typically to approximately 90% free feeding weight) and pre-exposed to the reward in their home cages 1-2 days prior to the first operant training session. All training sessions started with the delivery of a free reward. Reward delivery into the reward magazine was always paired with illumination of the receptacle light and a brief auditory click or tone throughout the experiment.

##### ***Stage I – Touch training***

Each session and trial began with delivery of a reward. Upon reward collection, the receptacle light was extinguished and a solid white rectangular stimulus (10cm long, 2.5cm high – centered horizontally – the bottom edge positioned approximately 4.5 cm above the grid floor) was presented on the screen (see Figure 4d). Touching the visual stimulus resulted in its removal from the screen and concurrent reward pellet delivery. Pellet collection immediately initiated the next trial (presentation of the rectangle stimulus on the screen). Each session lasted for either 100 trials or 60 minutes, whichever occurred first. Criterion for this stage was completing all 100 trials within 60 minutes and was typically achieved in 2-4 sessions.

##### ***Stage II – Two stimulus non-match training***

Upon reward collection of the first free reward, the receptacle light was extinguished and two solid white squares (3cm x 3cm, with the bottom edge located approximately 4.5cm above the grid floor) were presented on the touchscreen (see Figure 4d). Stimuli were positioned along the same horizontal row, with square separation varying from 2cm-6cm between different trials. After the animal touched either one of the two square stimuli (i.e., self-ordered), all stimuli were removed from the screen and a reward pellet was delivered. The first touch of an rSOWM trial is always correct. After reward collection, identical stimuli and positions were presented again on the screen. If the subject touched the same square

within the current trial ('incorrect response'), all stimuli were removed from the screen, the house light was turned on and a "dead fish" image was presented at the incorrect touch position for 5s to provide error feedback. After the 5s timeout period elapsed, the house light turned off, the "dead fish" stimulus was removed, and stimuli were displayed again to continue with the trial. If the subject touched an unvisited stimulus ('correct response'), all stimuli were removed from the screen and a reward was delivered. After touching both stimuli, the trial was deemed complete. Each trial was followed by an inter-trial interval (ITI) of 35s +/- 10s (5s increments) during which screen touches were without consequence. A new trial began immediately after the ITI period.

Sessions ended after completing 80 trials or after 60 minutes had elapsed, whichever occurred first. The criterion for completing this stage was set at achieving  $\geq 65\%$  perfect trials across 3 consecutive sessions. Criterion was typically reached in 5-12 sessions. We found that about 5% of subjects in a cohort may fail to reach criterion at this or later stages after such number of sessions.

### ***Stage III – Three stimulus non-match training***

Stage III is identical to *stage II* with the exception that 3 stimuli are presented in each trial (Figure 4d). The criterion for completing this stage was set at achieving  $\geq 26\%$  perfect trials across 3 consecutive sessions. Criterion was typically reached in 10-20 sessions. Note that chance performance on Stage III is 22.22%. After Stage III, mice were moved on to the full version of rSOWM (Figure 4a).

### ***Stage IV – Two and three stimulus non-match training***

*Stage IV* is a combination between *stages II* and *III*. Both trial types are pseudo-randomly presented across the session. The number of presentations of each trial type was matched and programmed to avoid more than three consecutive repetitions within a session.

### 4.3.3. rSOWM training protocol for mice

Similar to rats, mice received one daily session (5-6 sessions/week). Mice were food deprived according to national and institutional guidelines (typically to approximately 90% free feeding weight) and were pre-exposed to the milk reward in their home cages 1-2 days prior to the first operant training session. Mice were trained in a similar manner to rats with the exception of *Stage I – Touch training*.

*Stage I* in mice was divided into two substages. In *stage 1-A*, mice received a habituation session in order to be acclimated to the chamber and learn where rewards are delivered. This session began with a first larger 150µl reward. After reward collection, mice were given free 20µl rewards given every 10 seconds. Collection of the reward within the 10 second timeframe, speeded the reward delivery delay to 1 second. No visual stimuli were presented in this session. Reward delivery was paired with a tone and activation of the food receptacle light. The session ended after 60 minutes or when 100 rewards were collected, whichever occurred first. Criterion for this stage was collection of 40 rewards. In *stage 1-B*, mice were trained to touch the screen for rewards. Every trial consisted in the presentation of one white square stimuli randomly displayed in one of 5 positions (across the bottom horizontal area of the screen – see Figure 4d). The touch of the white square triggered a tone paired with the delivery of a reward, removal of the white square stimulus and activation of the food receptacle light. Blank touches to the rest of the screen were recorded but didn't trigger any response. ITI was set to 5 seconds. Criterion for this stage was collection of 60 rewards.

## 4.4. The rodent Delay and Reward Discounting (rDRD) task

### 4.4.1. rDRD task design

In rDRD, mice were required to nose-poke into the food magazine to initiate the session and, subsequently, each trial. This triggered the presentation of a pair of visual stimuli (gratings) on the screen that differed in size and luminance or gratings orientation. When one of the two images was touched, both stimuli were immediately removed from the screen and a sweetened milk reward was given with the activation of the food receptacle light. The time before delivery ('delay') and the milk reward size depended on the image selected (Figure 5a). A pool of 16 images varying on gratings orientation and overall image luminance and size were presented randomly and counterbalanced by screen side (left or right). One of the two image perceptual differences (gratings orientation and luminance) was used to inform about the reward size and the other was used for the delay (Figure 5b). This was also counterbalanced across mice. The final rDRD task was structured to include 4 different trial types: 1) "Forced Choice" trials in which only one image is presented, 2) "Same Delay" trials with images associated with identical delay, but different reward magnitude, 3) "Same Magnitude" trials with images associated with identical reward size, but different delays, and "Free Choice" trials which comprised a smaller-sooner (SS) and a larger-later (LL) image option. The inter-trial interval was set depending on the stimulus chosen so that the time between a stimulus touch and the next trial was constant at 20s across all trials. 1-2 training sessions of 60-90 min (or 150 trials maximum) were given daily.

rDRD delay discounting performance was assessed as the preference for SS over the more economically beneficial LL reward option across stimulus-pair presentations. Delay discounting choice behavior was modeled using multilevel logistic regression across individual data<sup>40</sup> in which sensitivity to reward magnitude ( $\beta_{reward}$ ) and delay ( $\beta_{delay}$ ) are considered separate contributors of choice. Logistic regression was used to estimate the hyperbolic parameter ( $k = \beta_{delay} / \beta_{reward}$ ) in trials in which the immediate delay is 0s<sup>41</sup>. See Statistical Analysis for further details.

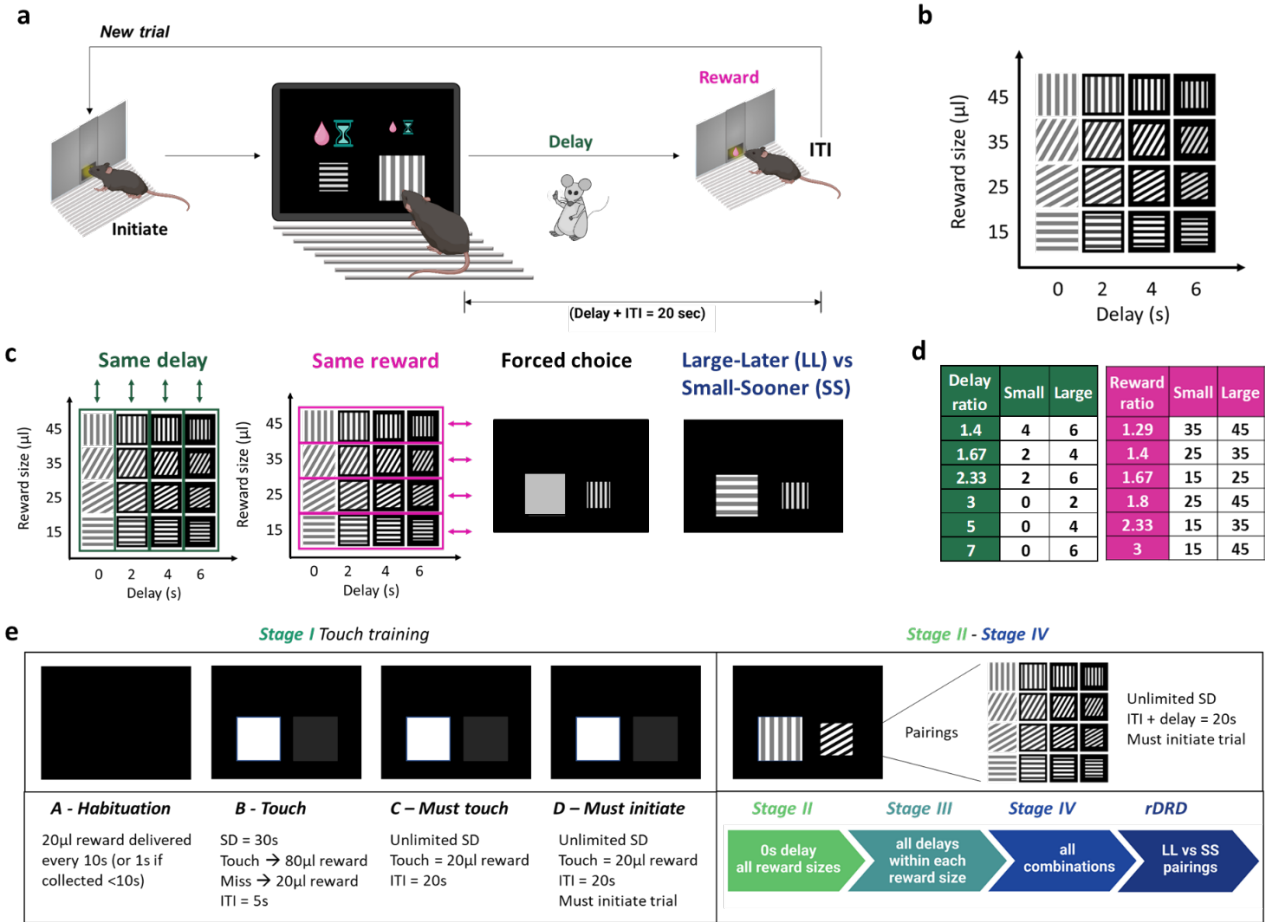


**Figure 5.** The rDRD behavioral paradigm. **a)** Schematic representation of the rDRD trials. **b)** Schematic representation of the visual stimuli used for the task. Gratings orientation and luminance/image size representing reward and delay magnitude, respectively, were counterbalanced across mice. **c)** rDRD trial types. Same delay trials had pairings of stimuli with the same delay. Same reward trials consisted in pairings of stimuli with the same reward size but different delays. Forced choice trials were pairings of any stimuli with an inactive grey square. Free choice trials, or Large-Later vs Small-Sooner trials, had pairings of stimuli with different delay and reward size such that the option with the larger reward had also a higher delay. **d)** Delay and reward ratio possibilities in each given trial. **e)** Schematic of the rDRD training procedure.

#### **4.4.2. rDRD training protocol for mice**

Animals learned the basic operation of the touchscreen operant chamber and the rDRD task structure through a series of training stages. Mice were food deprived according to national and institutional guidelines (typically to approximately 90% free feeding weight) and pre-exposed to the reward in their home cages 1-2 days prior to the first operant training session. 1-2 training sessions of 60-90 min (or 150 trials maximum) were given daily. All training sessions started with the delivery of a free reward. Reward delivery into the reward magazine was always paired with illumination of the receptacle light and a brief auditory click or tone throughout the experiment.

##### ***Stage I - Touch training***

Touch training was divided into 4 substages. In stage I-A, mice received a habituation session exactly as described in stage I-A for rSOWM. In stage I-B, mice were trained to touch the screen for reward. Each trial consisted in the presentation of one white square image randomly displayed in one of 2 fixed screen locations across the bottom horizontal area of the screen for 30 seconds (see Figure 5e). The touch of the white square triggered the delivery of a 80 $\mu$ l reward, three times more volume than the standard reward size we commonly use in mice. If 30 seconds passed and the white square was untouched, the stimulus image was removed and a 'free' 20 $\mu$ l reward was delivered. Blank touches to the rest of the screen were recorded but didn't trigger any response. ITI was set to 20 seconds. Criterion for this stage was set to collecting 60 rewards and was typically achieved in one session. Stage I-C was identical to I-B except that mice were required to touch the image in order to get a 20 $\mu$ l reward. No 'free' rewards were given. Instead the white square image stayed on the screen until it was touched. Criterion for this stage was also 60 rewards and was typically achieved in one session. Stage I-D was used to teach mice how to initiate a trial by poking in the reward magazine with their nose. After the 20-seconds ITI, the magazine light was lid and mice had to nose-poke into the magazine in order to start the next trial and get the white square image displayed on the screen. Everything else was unchanged. Criterion for this stage was 60 rewards and was typically achieved in 2-4 sessions.

##### ***Stage II – Reward size training (0s delay)***

Stages II and III were identical in session and trial structure as the full version of the rDRD task, with the only difference being the selection of images presented. In Stage II, only four images representing

0 delay across the four possible reward sizes were used in pairs. The ITI was set to 20 seconds. This stage was introduced to help them learn about the perceptual image characteristic representing reward magnitude and to expose the subjects to the different reward size options. Criterion for this stage was choosing the larger reward image option 70% of the trials and was typically achieved in 10 sessions.

The Two later cohorts of the 4 tested were trained in an additional intermediate stage before moving into Stage II. This consisted in choice pairings of the highest and lowest reward sizes only (Stage II-A). This was followed by additional pairings of the middle reward size options (Stage II-B). These two substages were introduced to help focus attention and help teach about the reward magnitude by introducing the most different reward size options first only.

### ***Stage III – Delay length training***

As in the full version of rDRD, all 16 images varying on gratings orientation and overall image luminance and size were presented in this stage. However, pairings within each trial were made by combining two images of the same delay. This stage was introduced to expose mice to the other 12 new images representing delays (i.e., 2, 4 and 6 seconds) but keeping the choice based on something already familiar, the perceptual characteristic representing reward magnitude. The inter-trial interval was set depending on the stimulus chosen so that the time between a stimulus touch and the next trial was 20s across all trials. Criterion for this stage was choosing the larger reward image option 70% of the trials and was typically achieved in 10 sessions.

The Two later cohorts of the 4 tested were trained in a variant of this stage (Stage III-B). In this stage, choices were limited to images representing 0 or 6 second delay across the different reward sizes. The other two reward sizes were introduced in the next and final version of the rDRD.

### ***Stage IV – Exposure to all image pairings***

All 16 images were displayed in random pairs. Preference analysis for each trial type was performed (Figure 5c): Same Delay, Same Reward, Forced Choice and Free Choice. After reaching criterion, mice moved on to the final version of the task in which the free choice trials were limited to LL-SS pairings.

## 4.5. Systemic pharmacology

**Modafinil.** Micronised Modafinil (*Lilly, UK*) was dissolved in a 10% (w/w) sucrose vehicle and administered orally to 39 rats (Cohorts 1 and 2) at a volume of 2ml/kg at doses of 8mg/kg, 16mg/kg, 32mg/kg (or vehicle, 10% (w/w) sucrose solution). Dose injections were administered according to a Latin Square design in which each animal received one different dose per session with at least one intervening baseline session. rSOWM behavioral testing started 30 minutes after administration of the drug.

**LSN2463359 [N-(1-methylethyl)-5-(pyridin-4-ylethynyl) pyridine-2-carboxamide].** LSN2463359 (*Lilly, UK*) was formulated as a suspension in a 1% (w/v) methylcellulose, 0.25% Tween 80 and 0.05% antifoam vehicle. LSN2463359 was administered orally (by gavage) at a volume of 2ml/kg, 30min before behavioral testing at doses of 1mg/kg, 2.5mg/kg, 5mg/kg, or vehicle solution. Dose injections were administered according to a Latin Square design in which each animal received one different dose per session with at least one intervening baseline session. rSOWM behavioral testing started 30 minutes after administration of the drug. A total of 20 rats (Cohort 1) were tested for this experiment.

**Ondansetron** (ondansetron hydrochloride dihydrate, *Sigma-Aldrich Inc. #Y0000218, USA*) 15 mice were injected (i.p.) at volumes of 1ml/kg at doses of 0.01, 0.1, 1mg/kg (or vehicle, 0.9% hydrochloride solution). Dose injections were administered according to a Latin Square design in which each animal received one different dose per session with at least 1 intervening baseline session. rDRD behavioral testing started 5 minutes after administration of the drug.

## 4.6. Intracranial pharmacology in mPFC

Bilateral stereotaxic implantation of microinfusion guide cannulas into the mPFC of rats was performed to enable local pharmacological administration. Rats were deeply anesthetized using isoflurane (5% for induction and 2.5% for maintenance) and secured in a stereotactic frame (David Kopf Instruments). The surgical area was shaved and disinfected with ethanol and a povidone-iodine solution and a midline incision was made along the scalp. A small craniotomy was performed bilaterally over the mPFC (coordinates: AP +3.2 mm, ML  $\pm$ 0.75 mm, DV -2 mm) using an electric drill. A 22-gauge stainless steel guide cannula was carefully inserted into the mPFC using a micromanipulator (David Kopf Instruments). To secure the cannula, micro-screws were inserted into the skull and the cannula was fixed in place with

dental cement. Following the procedure, rats were placed on a heating pad to maintain body temperature during recovery. Postoperative analgesia was administered with buprenorphine (0.05 mg/kg, s.c.) to manage pain. Rats were monitored for signs of distress, infection, or other complications and were given a week to rest before being exposed to behavioral testing. After surgery, mice received *ad libitum* access to food and water and were allowed to recover for at least one week. Body weight was monitored and food restriction was reinstated after recovery. Intracranial infusions were performed at least 10 days after surgery and rSoWM performance was confirmed to be back to the pre-surgery levels (~80% perfect responses in 2-stimuli trials and ~25% in 3-stimuli trials). On injection days, rSOWM performance was studied after 5 min of bilateral microinfusions of modafinil (0.1 or 1 µg/µl/side) and/or alpha-flupenthixol (0.5 µg/µl/side) directly into the mPFC. Micronised Modafinil (Lilly, UK) was dissolved in PEG:saline 5:95 (v/v) solution and at doses of 0 (PEG:saline 5:95 (v/v)) and 2µg/µl. Alpha-flupenthixol (Sigma-Aldrich, UK) was dissolved in saline solution and used at doses of 0 (saline) and 10 µg/µl. For pretreatment, alpha-flupenthixol or vehicle were infused bilaterally into the mPFC (coordinates: AP +3.2, L ±0.75, DV -4.5 from Bregma) over a 1-min period at a rate of 0.25 µl/min. Infusion of modafinil or vehicle were then performed 2 minutes following the pretreatment infusion. Behavioral testing began 5min after the final drug infusion. Drugs were administered according to a Latin Square design in which each group received one different dose per session with at least one intervening baseline session.

#### 4.7. Hippocampal lesions

Surgical procedures were based on publications on dorsal DG and CA3 manipulations for permanent, fibre-sparing inactivation (Lee and Kesner 2003). Animals lesioned for this study were donated by CA Oomen, who performed the surgeries (157) (Oomen CA. et al, 2015; Figure 6). Stereotaxic injection of vehicle (N=4 DG sham; N=4 CA3 sham) or neurotoxins colchicine (N=11 DG lesion) and ibotenic acid (N=10 CA3 lesion) were performed bilaterally. Rats were deeply anaesthetized with Isoflurane (5% for induction and 2.5% for maintenance) and placed in a stereotaxic frame. After a midline incision of the skin, localized holes were drilled in the skull above the injection sites. The stereotaxic coordinates (in mm) for DG lesions were AP -2.7 and -3.7 mm, ML ±1.6 and ± 2.5 mm, DV +3.6 and +3.1 mm, from bregma. For CA3 lesions, the three site coordinates were as follows: AP -2.5, -3.3, -4.1 mm, ML ±2.6, ±3.3 and ±4.2 mm, DV +3.2, +3.2, +3.1 mm. The same coordinates were used for the sham lesions, except no toxin was injected.

A 5ml Hamilton syringe, integrated within a microinjection pump, was connected to the stereotactic frame using H<sub>2</sub>O-filled polythene tubing. An injector with a 31-gauge needle was employed to administer various neurotoxins dissolved in saline. Injections were performed at a rate of 0.267 $\mu$ l/min. Colchicine (2.5 mg/ml), an excitotoxic neurotoxin that selectively targets dentate granule cells while sparing fibers, was administered into two sites per hemisphere (0.2 $\mu$ l/site) to permanently inactivate the DG. Ibotenic acid (6mg/ml), an excitotoxic, fiber-sparing neurotoxin with a preference for NMDA receptors was infused into three sites per hemisphere (0.2  $\mu$ l/site, 0.3 $\mu$ l/site, 0.6 $\mu$ l/site for the three sites targeting CA3, starting at the most anterior coordinate). Metacam (0.2 mg/kg, i.p.) was administered to manage post-operative pain and valium (5 mg/kg, i.p.), to prevent seizures was administered. Animals recovered in a heated cage in a darkened room. Food and water were provided ad libitum prior surgery and during the one week following surgery.

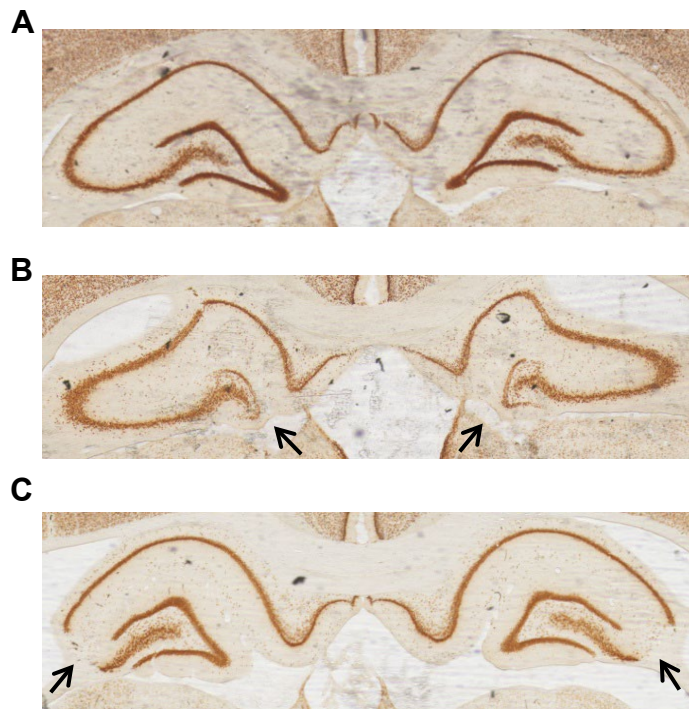


**Figure 6.** Photographs of representative sham lesions **(A)**, dentate gyrus lesions **(B)** and CA3 lesions **(C)**. The same coordinates were used for the sham lesions, except no toxin was injected.

## 4.8. Fiber-photometry recordings

Anesthesia was induced with 2–3% isoflurane (v/v) and maintained with 0.8%–1.5% isoflurane through a nose cone (Kent Somnosuite) mounted on a stereotaxic apparatus (David Kopf Instruments). Ketoprofen (10 mg kg<sup>-1</sup>, subcutaneous) was administered for pain management. The scalp was shaved and disinfected with ethanol and iodine solutions before exposing the skull. Craniotomies were drilled (0.4–0.5mm drill burr) above the target injection sites: mPFC (from Bregma, AP = +1.7, ML = +/- 0.3) and DLS (from Bregma, AP = +0.5, ML = +/- 2.5). AAV9 virus expressing GRAB sensors AAV9-hSyn-DA3h or GRAB AAV9-hSyn-5HT2h(5HT3.5)(WZ Biosciences) were injected unilaterally (400 nl) into the left or right mPFC (from Bregma, AP = +1.7, ML = +/- 0.3, DV = -2.5 from skull) or DLS (from Bregma, AP = +0.5, ML = +/- 2.5, DV = -2.8 from skull) via a glass pipette using a Nanoject II (Drummond Scientific). An optical fiber (400  $\mu$ m, 0.66 NA) with a ferrule connector was implanted at the 0.15mm above the viral injection site. The fiber was affixed to the skull surface using Metabond dental cement.

After surgery, mice received *ad libitum* access to food and water and were allowed to recover for at least one week. Body weight was monitored and food restriction was reinstated after recovery. The photometry rig (Doric Lenses Inc.) included a fluorescence optical mini cube with integrated photodetectors (iFMC6-G2\_IE(400-410)\_E1(460-490)\_F1(500-540)\_E2(555-570)\_F2(580-680)\_S, a blue LED (CLED\_560), and an LED driver (LEDD\_4). To record fluorescence signals, a beam of excitation light was emitted from an LED at 20–30  $\mu$ W, and the optical signals from the GRAB sensors were collected through optical fibers. The photometry data were collected with a sampling frequency of 1017 Hz. The recording fiber was bleached for ~30 minutes prior to the behavioral recordings to eliminate autofluorescence from the fiber. A control channel was constructed by smoothing the data trace and fitting an exponential to approximate any bleaching effects over the duration of the experiment. A fitted control channel was then obtained by fitting the control channel to signal channel using a least squares polynomial fit. The fluorescence response was analyzed using GuPPy (Sherathiya et al., 2021) and calculated as  $(\Delta F/F_0)$ , in which  $F_0$  is the fitted control channel and  $\Delta F$  is the fluorescence signal channel subtracted from the control channel. A Z-score is then calculated over the whole data stream by subtracting from each  $\Delta F/F_0$  data point the mean value and dividing by the standard deviation. The fluorescent signal for each behavioral event was plotted by aligning the Z-scored signal to the onset or offset of the behavior or task event.

Fiber photometry data were aligned to the behavioral data using TTL pulses (ABET TTL Adapter/Breakout Model 81510) generated by sensor events (e.g., trial initiation, reward collection) in the touchscreen chambers and sent to the Doric acquisition system.

## **4.9. Histology**

To evaluate the extent of DG and CA3 inactivation in rats (Results Chapter 1), immunohistochemical staining of neuronal nuclei (NeuN) was conducted. At the end of the study, rats were terminally anaesthetized with an overdose of sodium pentobarbital (Dolethal, Vetoquinol, UK) and subjected to transcardiac perfusion with 0.01 M PBS (pH 7.4) followed by paraformaldehyde solution (4% PFA in PBS). Brains were extracted and post-fixed in PFA for 18 hours. Coronal sections (60  $\mu$ m thick) were prepared using a sliding microtome, with prior cryoprotection in 20% sucrose solution and rapid freezing. Sections were collected in PB (0.01M, pH 7.4) and stained for NeuN using the primary antibody Mouse anti-NeuN (1:10.000; Vector) and secondary antibody biotinylated horse anti-mouse (1: 200; Vector). Diaminobenzidine (DAB) was used for chromogen development. Tissue damage was assessed in every sixth section of the dorsal hippocampus and drawn onto atlas diagrams.

For rats implanted with guide cannulae in Results Chapter 1, postoperative histological verification was performed to confirm accurate cannula placement. Rats were sacrificed and their brains were sectioned and stained with Cresyl Violet and verified with the guidance of an atlas (Paxinos and Watson, 2014) to assess the cannula's location within the mPFC.

## 4.10. Statistical analysis

1, 2 or 3-way ANOVA and repeated measures ANOVAs with behavioral task conditions (rSOWM: delay, stimuli number, separation; rDRD: delay, reward size) as within-subject factors, and experimental groups (e.g., pharmacological treatment, lesion, virus) as between-subject factors. Greenhouse-Geisser correction of the repeated-measures ANOVA was performed when Mauchly's test of sphericity was  $p < 0.05$ .

Appropriate post-hoc analysis (Šidák or Tukey-corrected, paired or unpaired *t*-tests) were conducted to evaluate performance across experimental groups. Linear and quadratic within-subjects contrasts were also used for the drug studies to determine trends in dose-response. Statistical analysis was performed using IBM SPSS Statistics for Windows 28.0.1.1 and GraphPad Prism 9. Differences were considered significant at  $p < 0.05$ .

For the rDRD task, a multilevel logistic regression model was used in which separate parameters are included to quantify the sensitivity to relative reward magnitude and the relative delay to reward. The approach simultaneously evaluates the discounting of individuals and groups of animals by examining choices between smaller sooner and larger later rewards. The data used in the logistic regression model were the Free Choice trial and included columns containing the SubjectID, Trial Number and Session Date, along with columns containing the SS Delay, SS Magnitude, LL Delay and LL Magnitude. The actual image chosen was coded in a Waited column, coded as 1 if the subject chose LL and as 0 if they chose SS. The following equation was implemented in the GLMER procedure in R (version 4.3.5) to estimate parameters for sensitivity to reward magnitude (associated with reward ratio) and the relative delay (associated with delay ratio) and were estimated for both individuals and the cohort. Where applicable, logistic regression was used to estimate the hyperbolic parameter ( $k = \beta_{\text{delay}} / \beta_{\text{reward}}$ ) in trials in which the immediate delay was 0s (146).

```
LogModel <- glmer(Waited ~ log(LL Magn/SS Magn) + log((LL Delay+1)/(SS delay+1)) - 1 +  
____(log(LL Magn/SS Magn) + log((LL Delay+1)/(SS Delay+1)) - 1 | Subject),  
data, family=binomial)
```

## 5. Results

### 5.1. CHAPTER 1 - The rodent Self-Ordered Working Memory paradigm in rats.

#### 5.1.1. rSOWM task validation and performance measures in rats.

Two cohorts of adult Lister hooded rats were used. Cohort 1 (n=22) underwent rSOWM training followed by behavioral probe manipulations as well as systemic pharmacological administration of putative cognitive enhancers modafinil and mGluR5 PAM (LSN2463359). Cohort 2 (n=19) was used to replicate the behavioral outcomes observed in the rSOWM paradigm, with and without systemic administration of modafinil. Following replication, cohort 2 also underwent intracranial surgical implantation of guide cannulas to enable central administration of modafinil, both alone and in combination with  $\alpha$ -flupentixol, to further replicate and investigate the potential mechanism by which modafinil exerted its enhancing effects on rSOWM.

Following pretraining *stages I-III* (see Methods), rats began rSOWM training *Stage IV* in which trials consisted in the presentation of two or three white-squares with a pre-stimulus delay set to 0 seconds. During the first eight sessions of *Stage IV*, performance steadily improved (main effect of session in % perfect trials:  $F(7, 147) = 16.17, p < 0.0001$ ) and errors/trial decreased monotonically:  $F(7, 147) = 15.30, p < 0.0001$ ) across both 2 and 3-stimuli trials (Figure 8). There was a main effect of stimulus number (% perfect trials:  $F(1, 21) = 406.8, p < 0.0001$ ; errors/trial:  $F(1, 21) = 718.2, p < 0.0001$ ). rSOWM performance remained significantly above chance levels ( $p < 0.0001$ ) from the second session onward in both trial conditions. Examination of the odds ratios relative to chance performance on 2- versus 3-stimulus trials indicated that odds of rats achieving perfect trials were not significantly different between trial types (2-stim =  $2.79 \pm 0.36$ ; 3-stim =  $2.33 \pm 0.39$ ;  $p > 0.05$ ).

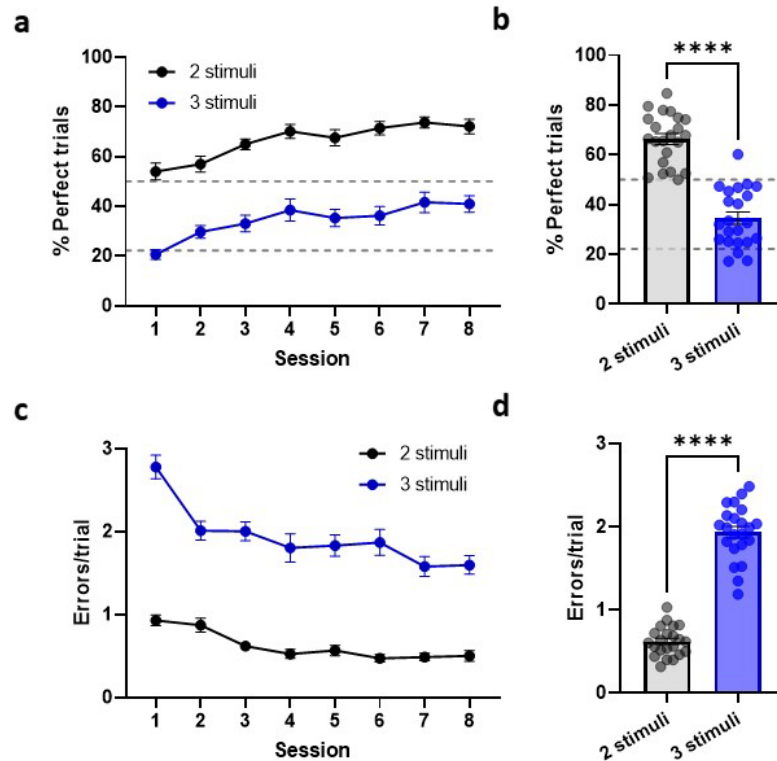


**Figure 8.** Training data on Stage IV of the rodent Self-Ordered WM (rSOWM) task. Data shows performance across eight training sessions on Stage IV. **a, b)** Mean performance analyzed as percentage of perfect trials **a**, for each of the sessions; **b**, averaged across the eight sessions. Discontinued horizontal lines indicate chance performance levels (50% perfect for 2-stimuli trials and 22.22% perfect for 3-stimuli trials. 2-way repeated measures ANOVA indicated there were significant main effects of session and stimulus number in both performance measures. **c, d)** Mean performance analyzed as number of errors divided by the total number of trials. **c**, for each of the sessions; **d**, averaged across the eight sessions. Discontinued horizontal lines indicate chance performance on % perfect trials corresponding to each trial type: 50% for 2-stimuli and 22.22% for 3-stimuli.

One of the key benefits of the touchscreen rSOWM task is its versatility and the wide range of parameter adjustments it supports. Self-sequencing, or the use of preferential ordering for organizing information that we need to remember, is a commonly employed strategy in everyday activities that require WM. To address whether self-sequencing the stimuli choices was important in rSOWM performance, we developed a task variant of the rSOWM, the Experimentally-Ordered Working Memory (EOWM) task (Figure 9). This variant eliminates the rats' ability to self-sequence their responses by requiring them to initiate each trial from a fixed location on the screen. In rEOWM, each trial began with only one white-square image presented. The image location where the stimulus was presented was

randomly assigned and counterbalanced for each session. Following the first touch, all stimuli images – either two or three, depending on the trial parameters - became visible, and the trial proceeded as usual in *Stage IV*. For this analysis, we compared mean performance across two sessions on rSOWM and two sessions of rEOWM task. Two-way repeated measures ANOVA revealed a main effect of task variant (% perfect trials:  $F(1, 21) = 40.87$ ,  $p < 0.0001$ ; errors/trial:  $F(1, 21) = 6.867$ ,  $p = 0.0160$ ) indicating a performance decrease in the rEOWM variant (Figure 9a). We hypothesized that sequencing could be more important in trials with 3 stimuli but we found no task variant x stimulus number interaction (% perfect trials:  $F(1, 21) = 1.308$ ,  $p = 0.2656$ ; errors/trial:  $F(1, 21) = 0.4526$ ,  $p = 0.5084$ ). We also investigated whether the distance separating the visual stimuli had any influence on their sequencing and, therefore, differential impacts on each task variant. We found no separation x task variant interaction in both trial types, 2-stimuli (% perfect trials:  $F(1, 21) = 0.5200$ ,  $p = 0.4788$ ; errors/trial:  $F(1, 21) = 0.08227$ ,  $p = 0.7771$ ) and 3-stimuli (% perfect trials:  $F(1, 21) = 0.01932$ ,  $p = 0.8908$ ; errors/trial:  $F(1, 21) = 2.786$ ,  $p = 0.1100$ ) trials (Figure 9b,c). Although task performance was decreased in the rEOWM task variant, rats still performed significantly above chance (2-stimuli trials  $p < 0.0001$ ; 3-stimuli trials  $p < 0.0001$ ) regardless of stimuli separation (2-stimuli, medium-separation  $p = 0.0003$ ; 2-stimuli hard-separation  $p = 0.0008$ ; 3-stimuli, medium-separation  $p < 0.0001$ ; 3-stimuli, hard-separation  $p = 0.0005$ ). These data comparing rEOWM and rSOWM indicate that self-sequence generation is an important strategy employed by subjects to enhance rSOWM performance; however, it is not essential for successful task execution.

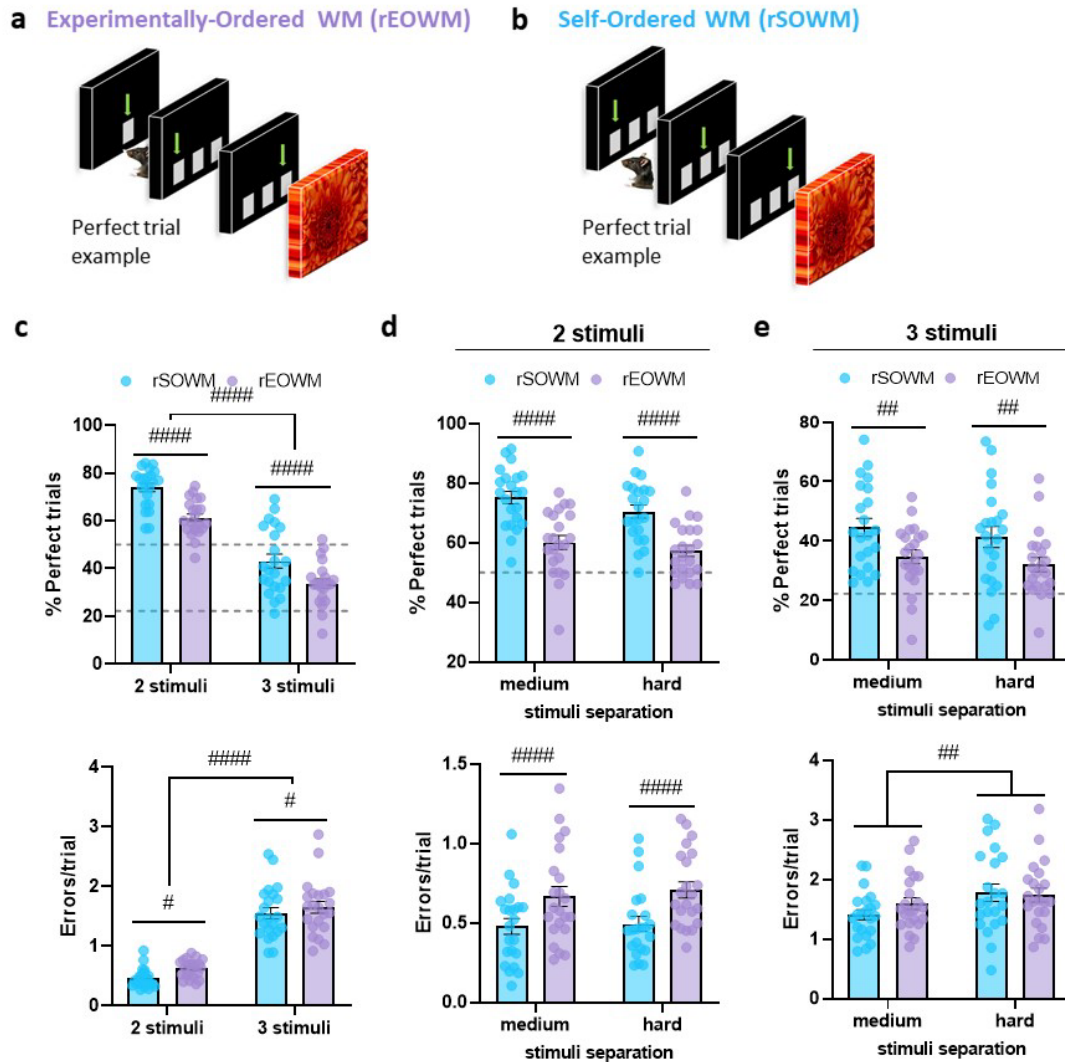


**Figure 9.** The rodent Experimentally-Ordered WM (rEOWM) task variant. **a)** Example illustration of a perfect 3-stimuli trial in the rEOWM task variant. Trial starts with the presentation of one visual stimulus choice. Rats are forced to touch that stimulus location in order to proceed and complete the trial. **b)** Example illustration of a 3-stimuli perfect trial in the rSOWM task. Rats can choose which visual stimulus to touch throughout the entire duration of the trial. **c)** Performance obtained across two consecutive sessions in each task. Top, performance is shown as % perfect trials; bottom, performance is shown as errors/trial. **d)** As in panel c, but analysis is broken down by stimulus number and separation between the stimuli presented - either medium (4.35cm) or hard (2.18cm). Discontinued horizontal lines indicate chance performance on % perfect trials corresponding to each trial type: 50% for 2-stimuli and 22.22% for 3-stimuli trials.

Following *stage IV*, rats were transitioned to the final version of rSOWM. These sessions were identical to those in *stage IV*, except that half of the trials included a 6-second delay between the first stimulus choice and subsequent presentations of the stimuli. We analyzed the impact of introducing trials with 6s delays by looking at the average performance across three consecutive sessions.

Performance in trials with 6s delays significantly decreased compared to trials with no delays (% perfect trials:  $F(1, 21) = 7.256$ ,  $p=0.0136$ ; errors/trial:  $F(1, 21) = 7.963$ ,  $p=0.0102$ ) (Figure 10a). Average group performance was significantly above chance across trial conditions (One sample  $t$  tests: 2 stimuli + 0s delay,  $p<0.0001$ ; 2 stimuli + 6s delay,  $p<0.0001$ ; 3 stimuli + 0s delay,  $p<0.0001$ ; 3 stimuli + 6s delay,  $p=0.0015$ ). Although separation between stimuli had a significant impact on task performance (% perfect trials: 2-stimuli  $F(2, 40) = 32.30$ ,  $p<0.0001$ , 3-stimuli  $F(1, 20) = 15.25$ ,  $p=0.0009$ ; errors/trial: 2-stimuli  $F(2, 40) = 33.63$ ,  $p<0.0001$ , 3-stimuli  $F(1, 20) = 15.03$ ,  $p=0.0009$ ) across difficulty conditions, there was no delay  $\times$  separation interaction (% perfect trials: 2-stimuli  $F(2, 40) = 0.1432$ ,  $p=0.8670$ , 3-stimuli  $F(1, 20) = 0.1481$ ,  $p=0.7044$ ; errors/trial: 2-stimuli  $F(2, 40) = 1.930$ ,  $p=0.1585$ , 3-stimuli  $F(1, 20) = 0.1583$ ,  $p=0.6949$ ) (Figure 10b,c), indicating that both task parameters had independent influence on task difficulty and outcome performance. We further analyzed performance in trials with three stimuli by looking at the number of errors made along the sequence of choices. The number of errors/trial made between the first correct touch and the second touch was similar to the error/trial rates observed in trials with 2-stimuli and lower than the number of errors/trial observed between the second and third correct choices (Figure 10d,e,f). As observed in Figure 10c, delay had a significant impact on performance, increasing the number of errors/trial before both the second ( $F(1, 20) = 9.616$ ,  $p=0.0056$ ) and third ( $F(1, 20) = 16.62$ ,  $p=0.0006$ ) correct choices. Stimuli separation, however, only significantly impacted the number of errors before the third ( $F(1, 20) = 10.83$ ,  $p=0.0036$ ) but not the second ( $F(1, 20) = 1.506$ ,  $p=0.2340$ ) choices. These data indicate that, as expected in trials with 3 stimuli, making a correct third touch choice was much more difficult than the second one.

Taken together, data from this cohort demonstrated that rSOWM performance was sensitive to common parametric manipulations that impact on WM performance in humans. Limiting self-sequencing strategies, decreasing the distance between stimuli and adding 6s delays each led to significant decreases in the number of perfect trials and increased the number of errors per trial.

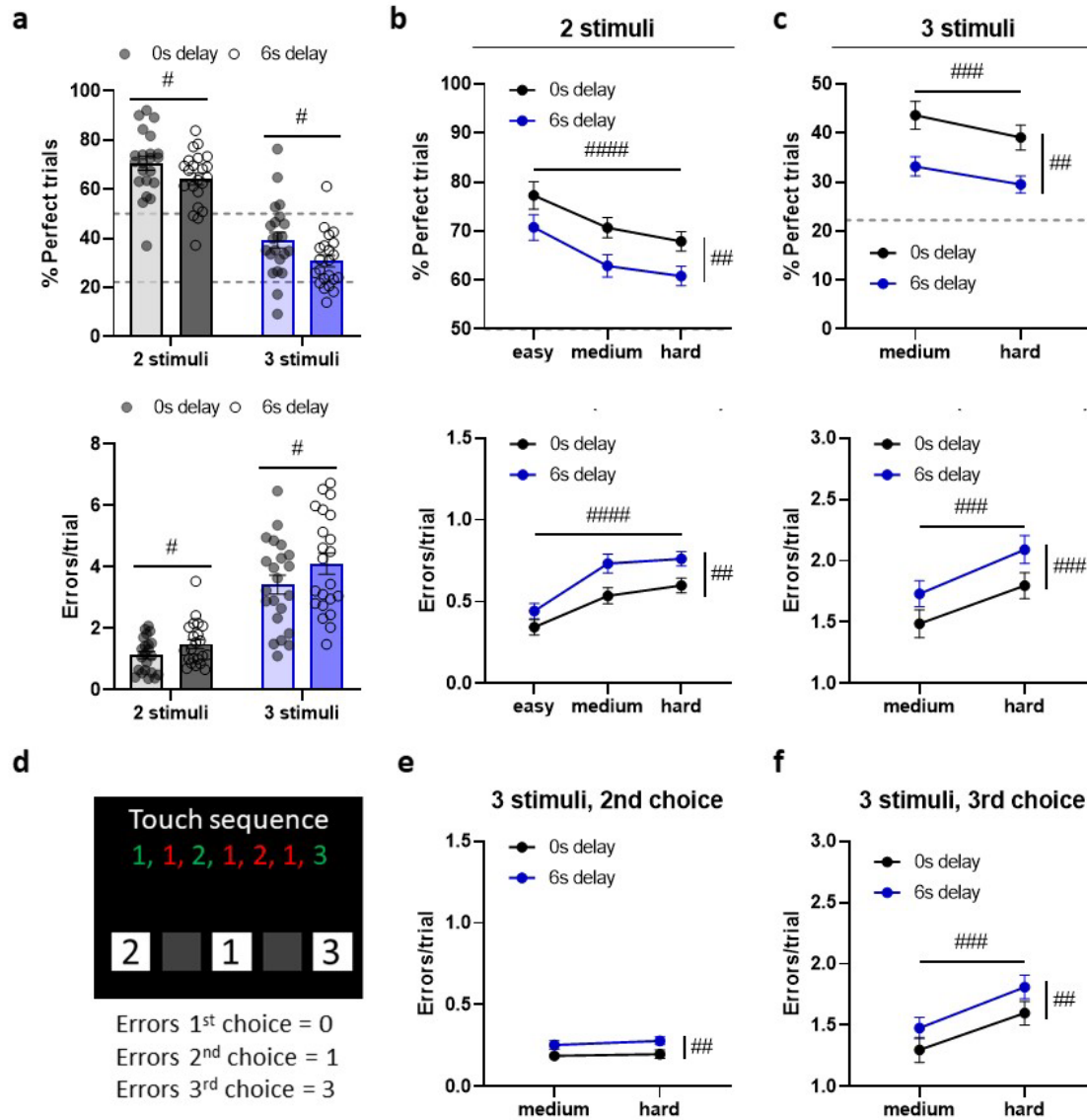


**Figure 10.** Average rSOWM performance across three sessions on the final training stage with delays. **a)** Average task performance, measured as % perfect trials (top) and number of errors/trial (bottom), comparing two delay conditions. **b)** Average task performance, measured as % perfect trials (top) and number of errors/trial (bottom), for 2-stimuli trials comparing the two delay conditions across the possible stimuli separations. **c)** Same as graph b, but presents data for 3-stimuli trials. **d)** Illustration of an example sequence of touches made to complete a trial. It is important to note that the first choice is always correct. In this example, the rat touched the middle stimulus, made an error, touched the left image, made three errors and finally touched the right and last stimulus. **e)** Same as c, but presents the number of errors/trial made before getting the second stimulus right. **f)** Same as e, but presents the number of errors/trial made between the second and third correct choices. Discontinued horizontal lines indicate chance performance on % perfect trials corresponding to each trial type: 50% for 2-stimuli and 22.22% for 3-stimuli trials.

### 5.1.2. rSOWM performance deficits following lesions to CA3 and dentate gyrus hippocampal subregions

A wealth of evidence has implicated a critical role for hippocampal function in spatial working memory. To investigate whether the rSOWM task was sensitive to disruption in hippocampal function, we assessed a cohort of 31 rats that had received either neurotoxic lesions targeting the CA3 (n=9) or DG (n=13) subregion of the dorsal hippocampus, in comparison to their respective sham controls (N=9). This cohort had been previously trained in a different touchscreen paradigm (157) and, thus, had already been trained on the basic principles required to perform a touchscreen task. We therefore began rSOWM training directly at *Stage II*. Rats were tested for four sessions at this *Stage II*, one session per day, on trials presenting two white-squared stimuli with no programmed delays (0 seconds) prior to presentation of the stimuli. Location and separation between the white squares were varied along the bottom row of the screen across trials to help avoid development of motoric stereotypic strategies (e.g., alternation). Performance across these four days was not different between the three groups (% perfect:  $F(2, 28) = 1.238$ ,  $p=0.3055$ ; errors/trial:  $F(2, 28) = 1.431$ ,  $p=0.2560$ ) (Figure 11b). All three groups were able to perform significantly above chance (50% perfect trials) (CA3  $p=0.0136$ , DG  $p=0.0003$  and SHAM  $p=0.0081$ ).

Rats were then moved on to *Stage III* in which the stimuli number was increased from two to three white squares. They were tested for 18 sessions. There was no difference in mean performance between the three experimental groups (% perfect:  $F(2, 28) = 0.2351$ ,  $p=0.7921$ ; errors/trial:  $F(2, 28) = 0.1235$ ,  $p=0.8843$ ) (Figure 11c). All three groups performed significantly above chance (22.222% perfect trials) (CA3  $p=0.0148$ , DG  $p=0.0034$  and SHAM  $p=0.0067$ ). To analyze whether there was a difference in learning and acquisition of this stage, we looked at their performance across 18 sessions grouped in blocks of 3 days. There was no significant session block x lesion group interaction ( $F(10, 140) = 1.012$ ,  $p=0.436$ ; errors/trial:  $F(10, 140) = 1.1782$ ,  $p=0.310$ ) indicating that none of the three groups improved their performance in a different manner than the rest of animals on this task stage (Figure 11d).

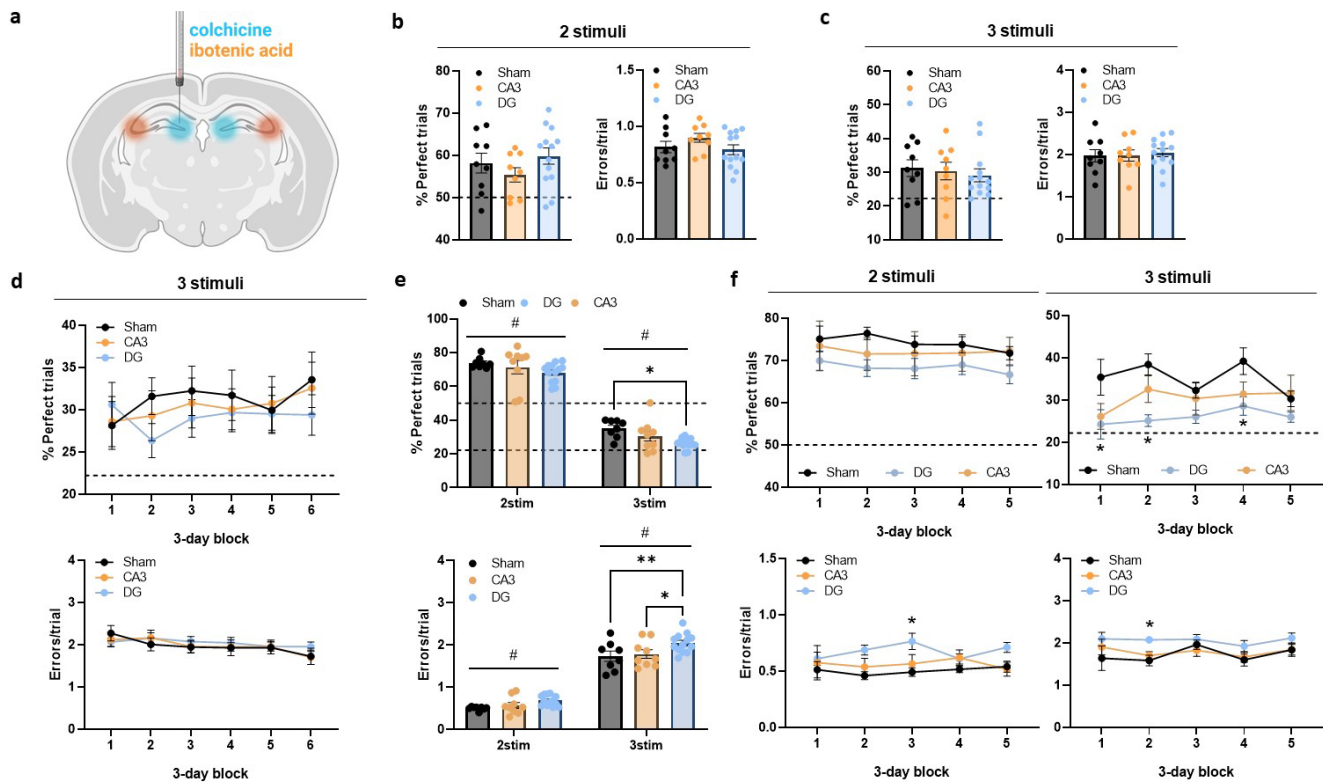


**Figure 11.** Effect of lesions targeting the DG or CA3 subregions of the hippocampus versus corresponding sham controls on rSOWM training and performance. **a)** Representation of the experimental surgical preparation of the intracranial injections of neurotoxins colchicine and ibotenic acid. Image generated with Biorender.com. **b)** Average performance in rSOWM Stage II shown in % perfect and number of errors per trial. SHAM controls and both DG and CA3 lesion groups were able to perform above chance levels and there was no significant difference between them. **c)** Average performance in rSOWM Stage III shown in % perfect and number of errors per trial. All three groups were able to perform above chance levels and there was no significant difference between them. **d)** Average performance in rSOWM Stage III shown in % perfect trials and number of errors per trial across blocks of 3 sessions. **e)** Average performance in rSOWM Stage IV shown in % perfect and number of errors per trial. # symbols indicate a main effect of lesion group in both performance measures. Sidak-corrected post-hoc tests indicated that DG-lesioned rats completed fewer perfect trials than the SHAM group (%perfect trials) and made more errors per trial than both SHAM and CA3 groups. **f)** Average performance in rSOWM Stage IV shown in % perfect trials and number of errors per trial across blocks of 3 sessions. \* indicates blocks in which DG animals performed significantly worse than SHAM rats. Discontinued horizontal lines indicate chance performance on % perfect trials corresponding to each trial type: 50% for 2-stimuli and 22.22% for 3-stimuli trials.

On *stage IV* (15 sessions), we observed a main effect of lesion group in both performance measures (% perfect:  $F(2, 27) = 4.247, p=0.0249$ ; errors/trial:  $F(2, 27) = 4.921, p=0.0151$ ) (Figure 11e). Post-hoc tests revealed that the DG group performed significantly worse than the SHAM group (Sidak-corrected pairwise comparisons: % perfect  $p=0.0229$ ; errors/trial  $p=0.0227$ ). Further analysis looking at performance for each trial type separately showed that the difference in performance between DG and SHAM animals was significant in trials with 3 stimuli (Sidak-corrected: % perfect  $p=0.0200$ ; errors/trial  $p=0.0296$ ). Repeated-measures ANOVAs of the three groups performance across 5 blocks of 3 days were also performed. There was no significant session block  $\times$  group interaction across any of the two trial types (2-stimuli trials - %perfect:  $F(8, 107) = 0.4077, p=0.9140$ ; errors/trial:  $F(8, 107) = 1.089, p=0.3764$ ; 3-stimuli trials - %perfect:  $F(8, 107) = 1.194, p=0.3094$ ; errors/trial:  $F(8, 107) = 0.4869, p=0.8632$ ). A main effect of lesion group was also present in this analysis for trials with 2 stimuli (errors/trial:  $F(2, 27) = 1.367, p=0.0397$ ) and 3 stimuli (% perfect:  $F(2, 27) = 5.547, p=0.0096$ ; errors/trial:  $F(2, 27) = 4.015, p=0.0298$ ). Post-hoc tests revealed that DG-lesioned rats had significantly fewer % perfect trials and more errors/trial than SHAM animals across the multiple session blocks (see Figure 11f). All three groups performed significantly above chance (2-stimuli trials: CA3  $p<0.0001$ , DG  $p<0.0001$  and SHAM  $p<0.0001$ ; 3-stimuli trials: CA3  $p=0.0019$ , DG  $p=0.0064$  and SHAM  $p=0.0017$ ).

After *Stage IV*, rats were moved to the final stage of the rSOWM task (3 sessions). All three lesioned groups were tested across trials varying in stimuli number (2 or 3) and delay (0 or 6 seconds), all combinations presented in a pseudo-random and counterbalanced manner. Introducing trials with 6s delays in Stage IV had a significant impact on rSOWM performance across all groups and trial types, decreasing the % of perfect trials (2-way ANOVA for 2-stimuli trials: (% perfect:  $F(1, 26) = 48.608, p<0.0001$  and 3-stimuli trials:  $F(1, 26) = 9.560, p=0.005$ ) (Figure 12a) and increasing the number of errors per trial (2-way ANOVA for 2-stimuli trials:  $F(1, 26) = 64.628, p<0.0001$  and 3-stimuli trials:  $F(1, 26) = 37.058, p<0.001$ ) (Figure 12b).

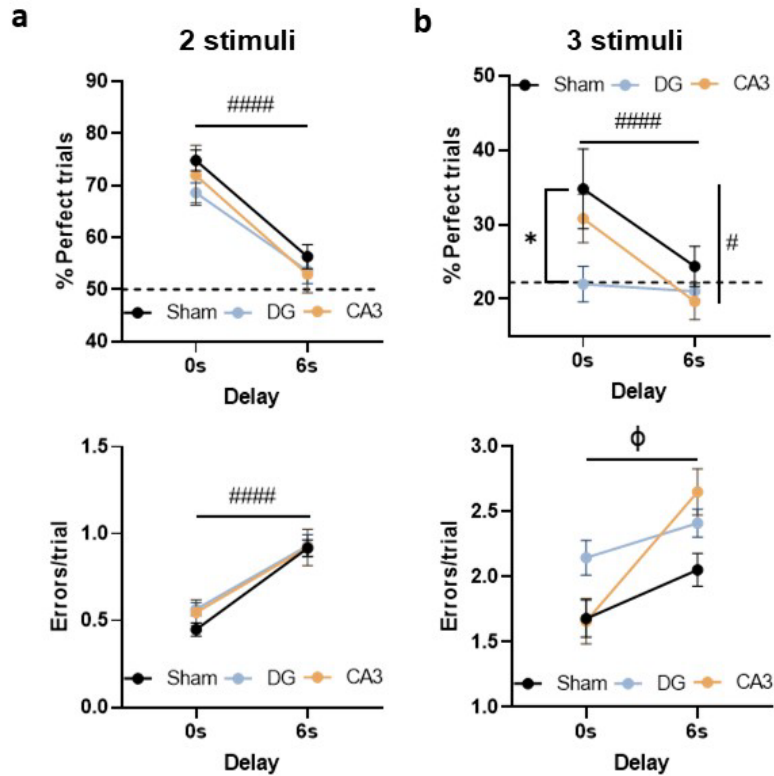


**Figure 12.** Effect of stimulus number and delay on hippocampal lesioned rats performing rSOWM Stage IV. **a)** Average task performance, measured as % perfect trials (top) and number of errors/trial (bottom), on trials with 2 stimuli comparing two delay conditions. **b)** Average task performance, measured as % perfect trials (top) and number of errors/trial (bottom), on trials with 3 stimuli comparing two delay conditions. # over horizontal lines indicate significant main effects of delay. # next to vertical lines indicate significant main effects of lesion group. \* indicates significant Sidak-corrected post-hoc comparisons  $p < 0.05$ . Discontinued horizontal lines in % perfect trials indicate chance performance levels (22.22% in 3-stimuli trials).

On trials with 2 stimuli, there were no significant delay x lesion group interactions (% perfect:  $F(2, 26) = 0.283$ ,  $p = 0.756$ ; errors/trial:  $F(2, 26) = 0.412$ ,  $p = 0.6664$ ) or main effects of lesion group (% perfect:  $F(2, 26) = 0.899$ ,  $p = 0.419$ ; errors/trial:  $F(2, 26) = 0.492$ ,  $p = 0.6173$ ). However, on trials with 3 stimuli we found a significant delay x lesion group interaction in errors/trial ( $F(2, 26) = 6.582$ ,  $p = 0.005$ ) (Figure 12b - bottom). We decomposed the interaction using one-way ANOVAs comparing group performance across delay conditions. We found significant simple main effects of lesion group in both delay conditions (errors/trial in 3-stimuli 0s delay:  $F(2, 26) = 3.716$ ,  $p = 0.038$ ; and 3-stimuli 6s delay:  $F(2, 26) = 4.255$ ,  $p = 0.025$ ). Further post-hoc comparisons uncovered that the CA3 lesion group performed differently compared to DG and SHAM animals depending on the delay condition. At 0s delay, CA3 lesioned rats had

a comparable number of errors/trial relative to the SHAM group (Tukey-corrected  $p=0.996$ ), while DG lesioned animals had significantly more errors/trial than CA3 and SHAM groups (Tukey-corrected  $p=0.063$ ). Conversely, on trials with 6s delay, CA3 lesioned rats were not significantly different from the DG group (Tukey-corrected  $p=0.415$ ) and both groups made more errors/trial than SHAM animals (Tukey-corrected  $p=0.020$ ) (Figure 12b - bottom).

There was no delay x lesion group interaction in 3-stimulus % perfect trials (3-stimuli trials:  $F(2, 26) = 2.064$ ,  $p=0.147$ ), but we observed a significant main effect of lesion ( $F(2, 26) = 4.167$ ,  $p=0.027$ ) (Figure 12b - top). Post-hoc comparisons indicated that DG lesioned animals performed significantly lower than SHAM animals in trials with 3 stimuli across both delays (Tukey-corrected  $p=0.0021$ ).

One sample t-tests revealed that all three groups were able to perform above chance in the easiest trial type (2 stimuli, 0s delay) and none in the hardest condition (3 stimuli, 6s delay). % perfect trials measure in DG and CA3 lesion groups was not significantly above chance levels on trials with delay (2 and 3 stimuli trials with 6s delay) ( $p>0.05$ ). % Perfect trials performance on SHAM rats was significantly higher than chance in trials with 2 stimuli, both with and without 6s delays, and very close to significant in the 3 stimuli, 0s delay condition ( $p=0.0512$ ). The CA3 lesioned group performed above chance only in trials without delays (2 and 3 stimuli trials with 0s delays). DG lesioned animals performed above chance only in the easiest 2 stimuli, 0s delay trial conditions.

Collectively, data on the final rSOWM stage suggest that the introduction of 6s delays lowered performance in all lesion groups. However, the effects were lesion and task parameter specific. Although all groups were able to perform above chance levels in the easiest parameter conditions (e.g., 2 stimuli, 0s delay), CA3 lesioned animals showed selective impairments in trial types with delays while the DG lesion group showed a more general impairment across all trial conditions except for the easiest 2-stimuli, 0s-delay trials.

### 5.1.3. rSOWM performance sensitivity to putative cognitive enhancers

#### 5.1.3.1. rSOWM task performance enhancement following systemic administration of modafinil

To investigate whether rSOWM task performance was sensitive to putative cognitive enhancer modafinil, we orally administered, through oral gavage, four different doses of modafinil thirty minutes prior to rSOWM testing. The three doses, 0, 8, 16 or 32 mg/kg, were assigned following a Latin Square design (**Figure 13b**). Each treatment session was followed by a rSOWM baseline session, with no modafinil administration, in order to ensure performance was back to baseline levels and to washout possible lingering effects of the drug (**Figure 13a**).

We first performed this experiment with Cohort 1 (n=21) and replicated the observed results in Cohort 2 (n=18). The two cohorts exhibited different levels of mean rSOWM performance across the entire pharmacological experiment (% perfect trials:  $F(1, 37) = 33.511$ ,  $p < 0.001$ ; errors/trial:  $F(1, 37) = 17.881$ ,  $p < 0.001$ ). This was likely due to Cohort 2 receiving fewer sessions on the full task of rSOWM and had initial baseline differences with lower % perfect trials ( $F(1, 37) = 23.110$ ,  $p < 0.001$ ) and higher errors/trial ( $F(1, 37) = 17.881$ ,  $p < 0.001$ ) compared to Cohort 1. It is important to note, however, that there was no cohort  $\times$  modafinil dose interaction ( $F(3, 111) = 1.199$ ,  $p = 0.313$ ) and that performance improvements after modafinil administration were observed in both cohorts.

In both cohorts, a significant linear improvement in %perfect with increasing modafinil dose ( $F(1, 37) = 4.651$ ,  $p = 0.038$ ) was observed only at the 3 stimuli and 6s delay trial conditions (**Figure 13f**). We hypothesized based on previous results in the human SWM task (158) that modafinil improvements would be observed particularly when task parameters were most challenging for working memory performance. Planned comparisons to test this hypothesis indicated that the highest 32mg/kg dose significantly increased %perfect ( $p < 0.05$ ) relative to vehicle treatment specifically at the 3 stimuli and 6s delay trial conditions. No significant effects of modafinil dose were observed in the average number of errors/trial ( $F(3, 111) = 0.065$ ,  $p = 0.978$ ) on nonperfect trials.

Comparison of performance after administration of vehicle with 'no-drug' session performance (average across the last 3 baseline sessions prior to drug treatment and the 3 wash-out days between drug sessions) revealed no significant differences, indicating that vehicle treatment alone did not exert

any significant effects on task performance (Figure 13c, d). As observed in regular testing sessions, significant main effects of delay, separation and stimuli number were observed on %perfect (all three parameters  $p < 0.001$ ) and errors/trial (all three parameters  $p < 0.001$ ) metrics, indicating that performance was sensitive to parameters affecting working memory load and similar to results observed in humans(158).

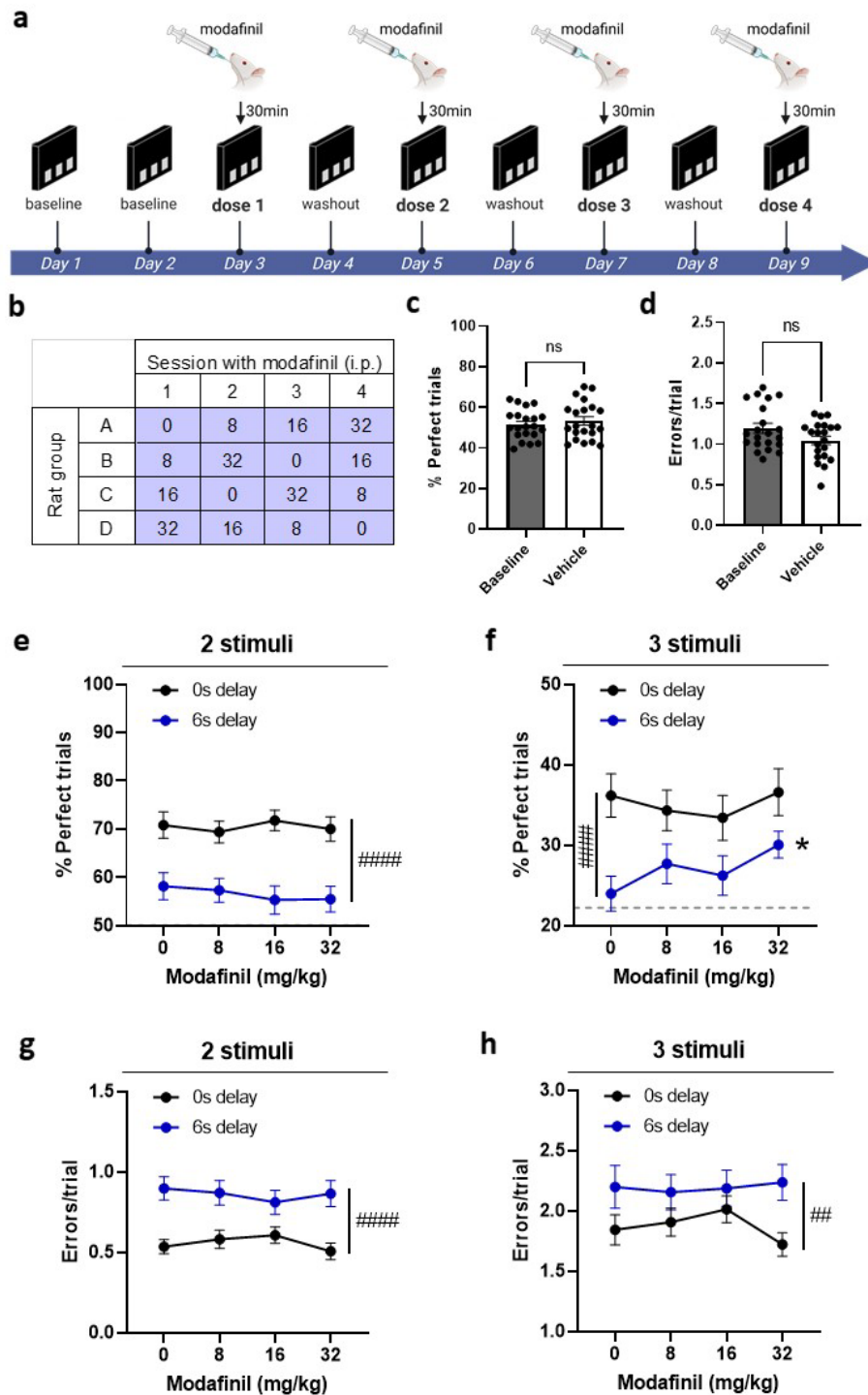


**Figure 13.** Effects of systemic administration of modafinil in rSOWM performance. **a)** Schematic timeline of the order and number of sessions used for the experiment. After two baseline sessions of rSOWM testing, rats received four sessions following modafinil systemic administration. Rats received a different dose of modafinil in each session. Sessions with injections were spaced by one more regular rSOWM session with no modafinil. Image created with

Biorender. **b)** Table illustrating the Latin square design used to assign the order of modafinil doses to the animals across the four sessions. **c,d)** Performance comparison between the two baseline sessions and the vehicle dose condition analyzed as % perfect trials (**c**) and errors/trial (**d**). **e)** Effect of modafinil dose on 2-stimulus % perfect trials across the two delay conditions. **f)** Effect of modafinil dose on 3-stimulus % perfect trials across the two delay conditions. **g)** Effect of modafinil dose on 2-stimulus number of errors/trial across the two delay conditions. **h)** Effect of modafinil dose on 3-stimulus number of errors/trial across the two delay conditions. # next to the vertical lines indicate significant main effects of delay. \* indicates a significant linear main effect of modafinil dose and significant post-hoc comparison between the vehicle and 32 mg/kg doses. Discontinued horizontal lines indicate chance performance on % perfect trials corresponding to each trial type: 50% for 2-stimuli and 22.22% for 3-stimuli trials.

### **5.1.3.2. rSOWM task performance enhancement following bilateral administration of modafinil into mPFC**

To further replicate and study the potential brain areas implicated in the observed task performance improvements observed after modafinil administration, 12 rats (Cohort 2) were surgically implanted with guide cannulas into both hemispheres on the prelimbic region of mPFC. After recovery and one week of rSOWM baseline training, implanted rats were administered four doses of modafinil across four sessions, spaced by at least one baseline session. The protocol used was identical to the one used for systemic administration of modafinil (Figure 13a) except that rSOWM testing commenced 5 minutes after modafinil intracranial infusions.

A significant improvement in perfect trials (%) was observed after bilateral microinfusion of modafinil in mPFC (Dose main effect:  $F [2, 22] = 5.597, p=0.011$ ) (Figure 14b). Within-subjects contrasts revealed a linear effect of modafinil dose ( $F [1, 11] = 6.712, p=0.025$ ) indicating that task improvements were stronger as the modafinil dose was increased. Tukey's Post-hoc comparisons revealed that the highest dose of modafinil increased %perfect trials compared to the low dose ( $p=0.02$ ) and vehicle conditions ( $p=0.01$ ).

Based on our results from the systemic administration of modafinil as well as previous results observed in the human SWM task (158), we tested the hypothesis that improvements in rSOWM performance after modafinil infusion would be observed when task parameters and working memory load were most difficult. Planned comparisons revealed that modafinil dose-dependently improved the

percent perfect on trials with 3 stimuli ( $F(2, 22) = 5.434$ ,  $p = 0.012$ ), where within-subject contrasts revealed a linear improvement of %perfect across ascending doses ( $F[1,11] = 10.56$ ,  $p = 0.008$ ) (Figure 14b). Post-hoc tests showed that microinfusion of  $2\mu\text{g}/\mu\text{l}$  ( $1\mu\text{g}/\text{side}$ ) modafinil significantly increased %perfect ( $p < 0.01$ ) relative to vehicle treatment.

Although modafinil infused in mPFC increased the number of perfect trials, it did not significantly reduce the number of errors (Modafinil dose effect on NPT-errors:  $F(2, 22) = 0.007$ ,  $p = 0.994$ ; Modafinil dose effect on errors/trial:  $F(2, 22) = 0.845$ ,  $p = 0.443$ ).

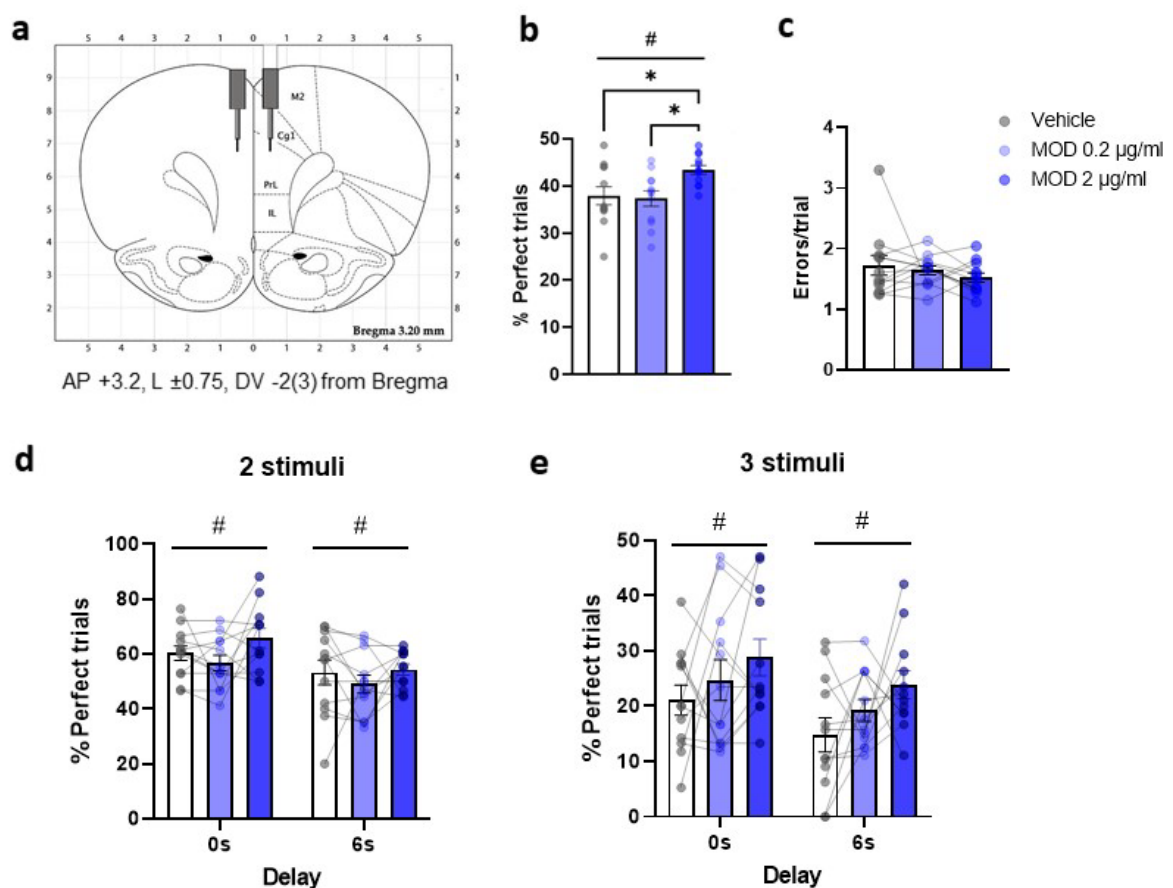


**Figure 14.** Effects of bilateral microinfusions of modafinil in prelimbic mPFC. **a)** Schematic illustration of the placement of the guide cannula in the rat medial prefrontal cortex (mPFC). Representation of a coronal section with the target coordinates for cannula implantation. Adapted from Paxinos and Watson (1997). **b)** Effects of bilateral infusion of modafinil in %perfect trials (left: across all trial conditions and task difficulty levels; right: performance is split by delay and stimuli number conditions). There was a significant linear main effect of modafinil dose. # indicate significant linear main effects of modafinil dose. Significant Tukey-corrected Post-hoc comparisons between

modafinil doses are shown as \* $p < 0.05$  \*\* $p < 0.005$ . **c)** Effects of bilateral infusion of modafinil in the number of errors/trial was not significant.

Although the precise mechanisms of the nootropic effects of modafinil are unknown, it is well known that modafinil can dose dependently stimulate monoamine systems such as dopamine in the brain. Thus we performed bilateral microinfusion of d1/d2 receptor antagonist,  $\alpha$ -flupenthixol, into the mPFC to test whether we could block the performance improvements of modafinil. Four combinations of the two drugs modafinil and  $\alpha$ -flupenthixol (vehicle + vehicle, vehicle + modafinil,  $\alpha$ -flupenthixol + vehicle and  $\alpha$ -flupenthixol + modafinil) were administered following a Latin Square design (Figure 15a). Replicating the previous microinfusion experiment, we observed a rSOWM performance improvement following modafinil microinfusions. Although %perfect trials were not significantly different between the four drug combinations (Figure 15b), a main effect of drug combination ( $F [3, 21] = 7.123, p = 0.002$ ) and a significant interaction of modafinil x  $\alpha$ -flupenthixol were observed in the number of errors/trial ( $F [1, 7] = 14.557, p = 0.007$ ) (Figure 15c). We further decomposed the interaction by repeated-measures ANOVAs across the conditions with  $\alpha$ -flupenthixol present or absent separately. Modafinil significantly reduced the number of errors/trial when comparing the two drug combinations without  $\alpha$ -flupenthixol (Modafinil main effect:  $F [1, 8] = 10.126, p = 0.013$ ) and not across drug combinations with  $\alpha$ -flupenthixol present (Modafinil main effect:  $F [1, 7] = 1.397, p = 0.276$ ).

Based on our hypothesis that modafinil would improve performance on the most difficult trial types, we broke down the analysis across stimuli number. The significant modafinil x  $\alpha$ -flupenthixol interaction ( $F [1, 7] = 7.851, p = 0.026$ ) was observed on the 3-stimulus trial condition and not the 2-stimulus (Figure 15d-e). Post-hoc comparisons indicated a significant difference between the vehicle + modafinil and  $\alpha$ -flupenthixol + modafinil groups (Tukey-corrected  $p < 0.01$ ). Modafinil significantly reduced the number of errors when infused alone but not when co-infused with  $\alpha$ -flupenthixol. No significant differences on the error rate were found when  $\alpha$ -flupenthixol was infused (with vehicle or with modafinil) relative to the vehicle + vehicle treatment condition.

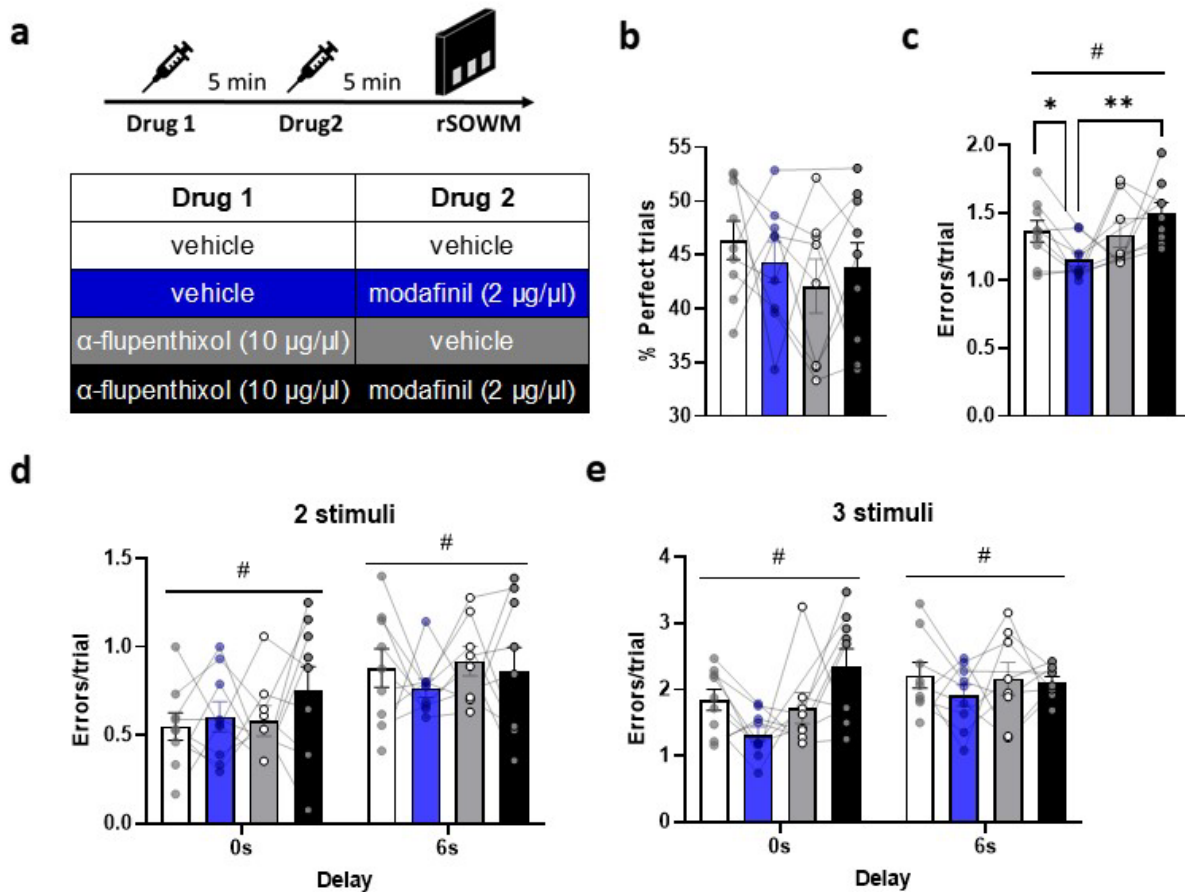


**Figure 15.** Bilateral microinfusion of d1/d2 receptor antagonist, alpha-flupenthixol, in mPFC blocks the enhancing effects of modafinil in rSOWM. **a)** Illustration of the microinfusion protocol used to assess whether prefrontal d1-d2 receptors are involved in the rSOWM performance improvements produced by modafinil. In this experiment,  $\alpha$ -flupenthixol or vehicle were infused bilaterally into the mPFC (coordinates: AP +3.2, L  $\pm$ 0.75, DV -4.5 from Bregma) over a 1-min period at a rate of 0.25  $\mu\text{l}/\text{min}$ . After 5 minutes, modafinil or vehicle were infused in the same manner. Behavioral testing began 5min after the second drug microinjection. **b)** Effects of bilateral microinjection of  $\alpha$ -flupenthixol and modafinil on %perfect trials. **c)** Effects of bilateral microinjection of  $\alpha$ -flupenthixol and modafinil on number of errors/trial (left: across all trial conditions and task difficulty levels; right: performance is split by delay and stimuli number conditions). Modafinil significantly reduced the number of errors/trial metric when injected with vehicle. Infusions with  $\alpha$ -flupenthixol blocked the enhancing effects of modafinil. # indicate significant main effects of drug combination. Significant Tukey-corrected Post-hoc comparisons between modafinil doses are shown as \*  $p < 0.05$  \*\*  $p < 0.01$ .

### **5.1.3.3. rSOWM task sensitivity to mgluR5 positive allosteric modulator (LSN2463359)**

To investigate whether mgluR5 positive allosteric modulator (PAM) LSN2463359 exerting cognitive enhancing effects on rSOWM performance, we administered four different doses of LSN2463359 thirty minutes prior to rSOWM testing via oral gavage. The four doses, 0, 1, 2.5 or 5 mg/kg, were assigned to the rats following a Latin Square design (Figure 16b). Sessions performed after injection of LSN2463359 were spaced by baseline rSOWM sessions to washout any possible leftover effects that could impact performance (Figure 16a). This experiment was conducted on 20 rats (Cohort 1). One rat was excluded from the study due to complications encountered during oral gavage in two of the four sessions.

In contrast to the effects of modafinil, no significant effects of LSN2463359 drug were seen on rSOWM performance (Figure 16e-h) both in 2-stimuli trials (% perfect:  $F(3, 54) = 0.7382$ ,  $p=0.5339$ ; errors/trial:  $F(3, 54) = 1.244$ ,  $p=0.3029$ ) and 3-stimuli trials (% perfect:  $F(3, 54) = 0.6712$ ,  $p=0.5734$ ; errors/trial:  $F(3, 54) = 0.7880$ ,  $p=0.5058$ ). Task performance after vehicle was not significantly different from session performance on 'no-drug' days (Figure 16c, d). As observed in sessions with no pharmacological intervention, significant main effects of delay ( $F(1, 19) = 24.23$ ,  $p<0.001$ ), number of stimuli ( $F(1, 19) = 192.59$ ,  $p<0.001$ ) and separation ( $F(1, 19) = 37.68$ ,  $p<0.001$ ) were observed on % perfect trials.

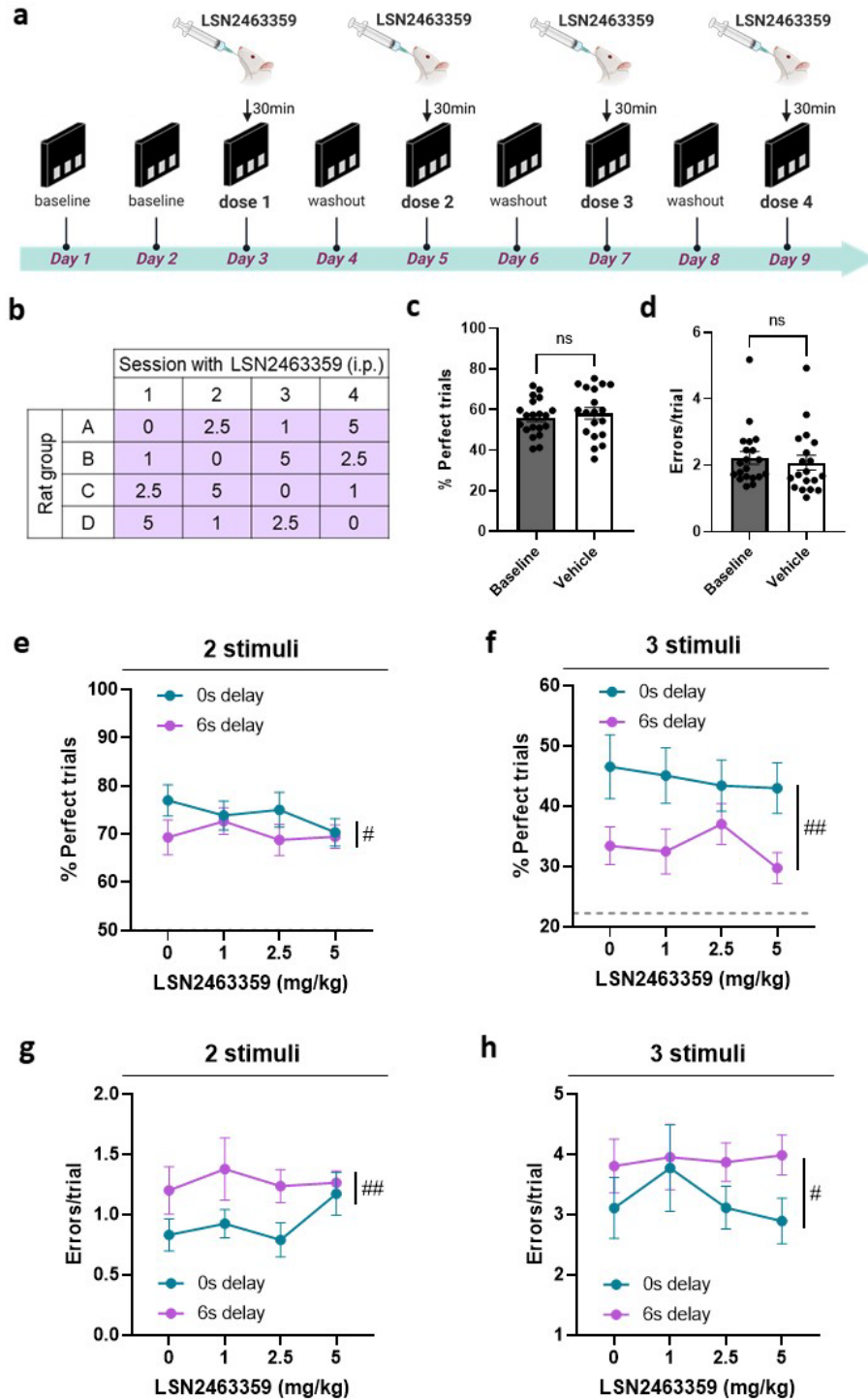


**Figure 16.** Effects of systemic administration of mGluR5 PAM (LSN2463359) in rSOWM performance. **a)** Schematic timeline of the order and number of sessions used for the experiment. After two baseline sessions of rSOWM testing, rats received four sessions following systemic administration of LSN2463359. Rats received a different dose of

LSN2463359 in each session following a Latin square design. Sessions with LSN2463359 were spaced by one or more regular rSOWM baseline sessions with no drug. Image created with Biorender. **b)** Table illustrating the Latin square design used to assign the order of LSN2463359 doses to the animals across the four sessions. **c, d)** Performance comparison between the two baseline sessions and the vehicle dose condition analyzed as % perfect trials (**c**) and errors/trial (**d**). **e)** Effect of LSN2463359 dose on 2-stimulus % perfect trials across the two delay conditions. **f)** Effect of LSN2463359 dose on 3-stimulus % perfect trials across the two delay conditions. **g)** Effect of LSN2463359 dose on 2-stimulus number of errors/trial across the two delay conditions. **h)** Effect of LSN2463359 dose on 3-stimulus number of errors/trial across the two delay conditions. # next to the vertical lines indicate significant main effects of delay. Discontinued horizontal lines indicate chance performance on % perfect trials corresponding to each trial type: 50% for 2-stimuli and 22.22% for 3-stimuli trials.

## 5.2. CHAPTER 2. The rodent Self-Ordered Working Memory paradigm in mice.

### 5.2.1. rSOWM task validation and performance measures in mice.

Three cohorts of C57BL6J mice were used for experiments in chapter 2. The first cohort of 15 mice was trained as detailed in the methods section 4.3.3. After receiving one habituation session and one session on touch training, mice were tested on the easiest parameter of the task (*Stage II*), 2 stimuli with 0s delay, for 42 sessions. Performance during this stage improved steadily across training sessions with increasing percentage of perfect trials (Greenhouse-Geisser correction for sphericity:  $F(4.756, 66.577) = 19.439$ ,  $p < 0.001$ ) and decreasing numbers of errors/trial (Greenhouse-Geisser correction for sphericity:  $F(4.195, 58.731) = 17.954$ ,  $p < 0.001$ ). Although mice were kept at this stage for 42 sessions to maximize task exposure and learning, average group % perfect trials reached stable performance levels with significance above chance from sessions 16-18 (3-day block 6,  $p < 0.01$ ) (Figure 17a).

Mice were then transitioned to *Stage III* and tested for 4 sessions. rSOWM group performance was significantly above chance in each of the 4 sessions ( $p < 0.0001$ ) and individual or group performance did not change significantly across the first four sessions (% perfect trials:  $F(3, 33) = 1.383$ ,  $p = 0.265$ ; errors/trial:  $F(3, 33) = 2.067$ ,  $p = 0.124$ ) (Figure 17b). Thus, all animals were shifted to *Stage IV* which includes randomly presented trials having 2 or 3 stimuli. There was a session block x stimuli number significant interaction in both performance measures (% perfect trials:  $F(8, 112) = 4.661$ ,  $p < 0.0001$ ; errors/trial:  $F(8, 112) = 3.987$ ,  $p = 0.0003$ ) as well as significant main effects of stimuli number (% perfect trials:  $F(1, 14) = 310.0$ ,  $p < 0.0001$ ; errors/trial:  $F(1, 14) = 702.8$ ,  $p < 0.0001$ ) and session block (% perfect trials:  $F(8, 112) = 3.990$ ,  $p = 0.0003$ ; errors/trial:  $F(8, 112) = 5.553$ ,  $p < 0.0001$ ). Further analysis across each stimuli number condition indicated that, during the 42 sessions, performance on 2-stimuli trials remained stable over 70% perfect trials and was significantly above chance across all sessions ( $p < 0.01$ ). Performance at 3-stimuli, however, improved across training sessions and remained higher than chance levels after session 12 (Figure 17c). As in the SOWM task in rats, when odds ratios of performance relative to chance were compared for 2 versus 3 stimulus trials, the odds achieving a perfect trial were both highly above chance but not significantly different from each other (2-stim OR =  $2.84 \pm 0.37$ ; 3-stim OR =  $2.13 \pm 0.46$ ,  $p > 0.05$ ).

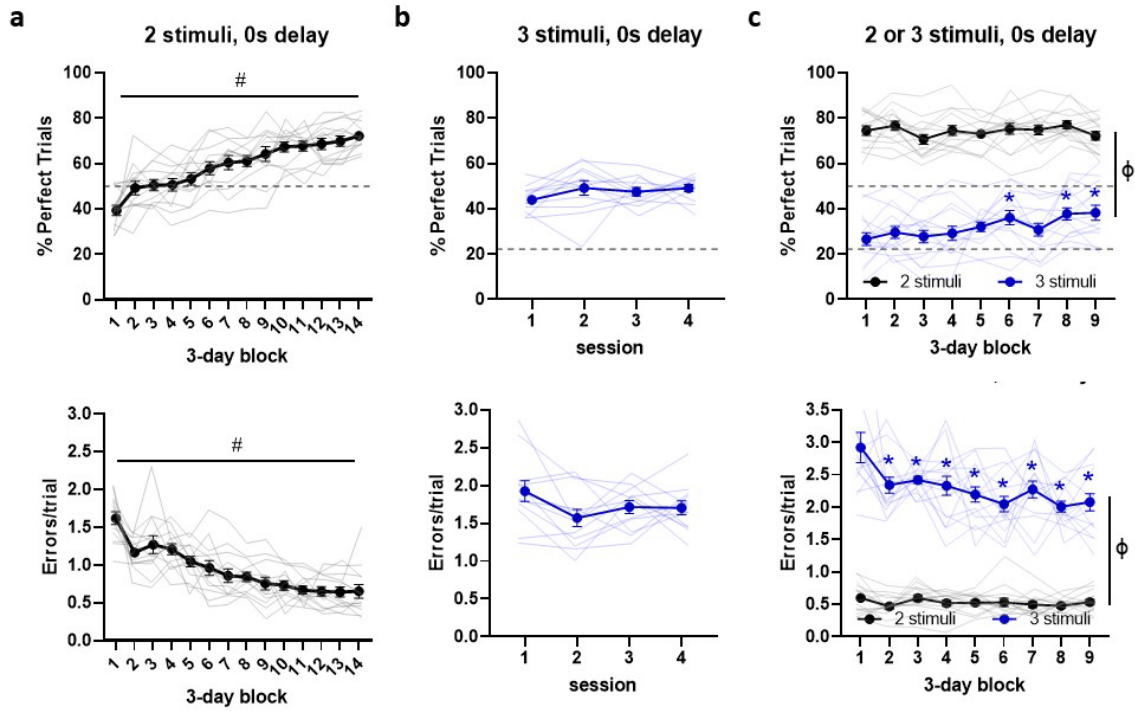


**Figure 17.** rSOWM performance of mice during training and acquisition. **a)** Mean rSOWM performance, measured as % perfect trials (top) and number of errors per trial (bottom), during Stage II of training. Data shows performance across 42 sessions grouped in 3-day blocks. Trial parameters were set to 2 stimuli and 0s delay for all trials. # indicates a main effect of session with performance improving linearly with training. Average performance was significantly above 50% chance after block 6 ( $p < 0.01$ ). **b)** Mean rSOWM performance, measured as % perfect trials (top) and number of errors per trial (bottom), during Stage III of training. Data shows performance across 4 sessions. Trial parameters were set to 3 stimuli and 0s delay for all trials. Performance was stable across the four sessions and significantly above chance ( $p < 0.0001$ ). **c)** Mean rSOWM performance, measured as % perfect trials (top) and number of errors per trial (bottom), during Stage IV of training. Data shows performance across 27 sessions. Sessions consisted on trials with either 2 or 3 stimuli and 0s delays.  $\Phi$  indicate significant stimulus number x session block interaction. Performance at 2-stimuli trials was stable across time and significantly above chance from the first session ( $p < 0.0001$ ). Performance on 3-stimuli trials improved over time and was significantly above chance (22.22% perfect 3-stimuli trials) on blocks 2 and 4 to 9 ( $p < 0.05$ ). \*  $p < 0.05$  Tukey-corrected post-hoc comparisons between block 1 and each subsequent block. Discontinued horizontal lines indicate chance performance on % perfect trials corresponding to each trial type: 50% for 2-stimuli and 22.22% for 3-stimuli.

We next modified the program to include trials with 2, 3 and 4 visual stimuli with no delays. Performance significantly decayed in trials in which mice had to remember 3 previously visited locations on the screen (Figure 18a,b). Percentage of perfect trials significantly decreased as the number of stimuli increased ( $F(2, 45) = 380.2$ ,  $p < 0.0001$ ) and the number of errors was significantly increased ( $F(2, 26) = 133.4$ ,  $p < 0.0001$ ). Sidak-corrected comparisons indicated that performance across stimuli number conditions was significantly different on both performance measures, % perfect trials and errors/trial (all  $p < 0.0001$ ). One sample t-tests comparing mean performance, across 49 sessions given with this same task parameters, with chance % perfect trials performance indicated that mean performance across each trial type was significantly above chance in all three conditions: 2stim  $p < 0.0001$ , 3stim  $p < 0.0001$  and 4stim  $p = 0.0167$ . Session-by-session analysis revealed that mice were able to perform significantly above chance starting on the sixth session (2stim  $p = 0.0007$ , 3stim  $p = 0.0168$  and 4stim  $p = 0.0291$ ).

Once performance was stably above chance across all three stimuli number conditions, pre-stimulus delays (3s) were added randomly on 50% of the trials for each stimulus number for six sessions. Both difficulty parameters, stimuli number and delay, impacted performance by reducing % perfect trials (2-way RM ANOVA: stimuli number  $F(2, 26) = 133.4$ ,  $p < 0.0001$ ; delay ( $F(1, 13) = 4.818$ ,  $p = 0.0469$ ) and increasing the number of errors (2-way RM ANOVA: stimuli number  $F(2, 26) = 133.4$ ,  $p < 0.0001$ ; delay  $F(1, 13) = 4.818$ ,  $p = 0.0469$ ) (Figure 18c,d). One sample t-tests revealed that performance was significantly above chance levels for all trial type combinations: 2-stimuli + 0s delay  $p = 0.0002$ , 2-stim + 3s delay  $p = 0.0006$ , 3-stimuli + 0s delay  $p = 0.0015$ , 3-stimuli + 3s delay  $p = 0.0003$ , 4-stimuli + 0s delay  $p = 0.0443$ , and 4-stimuli + 3s delay  $p = 0.0149$ .

Delays were further increased from 3 to 6 seconds for the next 16 sessions. A significant stimuli number x delay interaction ( $F(2, 26) = 5.899$ ,  $p = 0.0077$ ) was observed in the % perfect trials obtained on these conditions (Figure 18e,f). Significant main effects of stimuli number ( $F(2, 26) = 663.3$ ,  $p < 0.0001$ ) and delay conditions ( $F(1, 13) = 11.45$ ,  $p = 0.0049$ ) were also observed in % perfect trials. Further mean comparisons revealed that delay had significant decreases in % perfect trials in trials with 2 (Sidak-corrected:  $p < 0.0001$ ) and 3 (Sidak-corrected:  $p = 0.0077$ ) stimuli but was not significant in trials with 4 (Sidak-corrected:  $p = 0.7189$ ). Group mean % perfect trials performance was significantly above chance levels in trials with 2-stimuli, at 0 and 6s delays (both  $p < 0.001$ ), trials with 3-stimuli with 0s delays ( $p = 0.0149$ ) but not on the most difficult trial conditions with 3 stimuli and 6s delays ( $p = 0.0817$ ) or 4 stimuli with or without delays ( $p = 0.1328$  and  $p = 0.2751$ , respectively). There was a main effect of stimuli number

( $F(2, 26) = 175.2$ ,  $p < 0.0001$ ) but no significant main effect of delay on the number of errors/trial performance metric ( $F(1, 13) = 1.637$ ,  $p = 0.2231$ ). The results indicated that when 6s pre-stimulus delay trials were added, general performance at 4 stimuli (with or without delays) deteriorated to levels not significantly above chance. As our goal was to further investigate delay effects on performance, we proceeded on experiments using only 2 or 3 stimuli number.

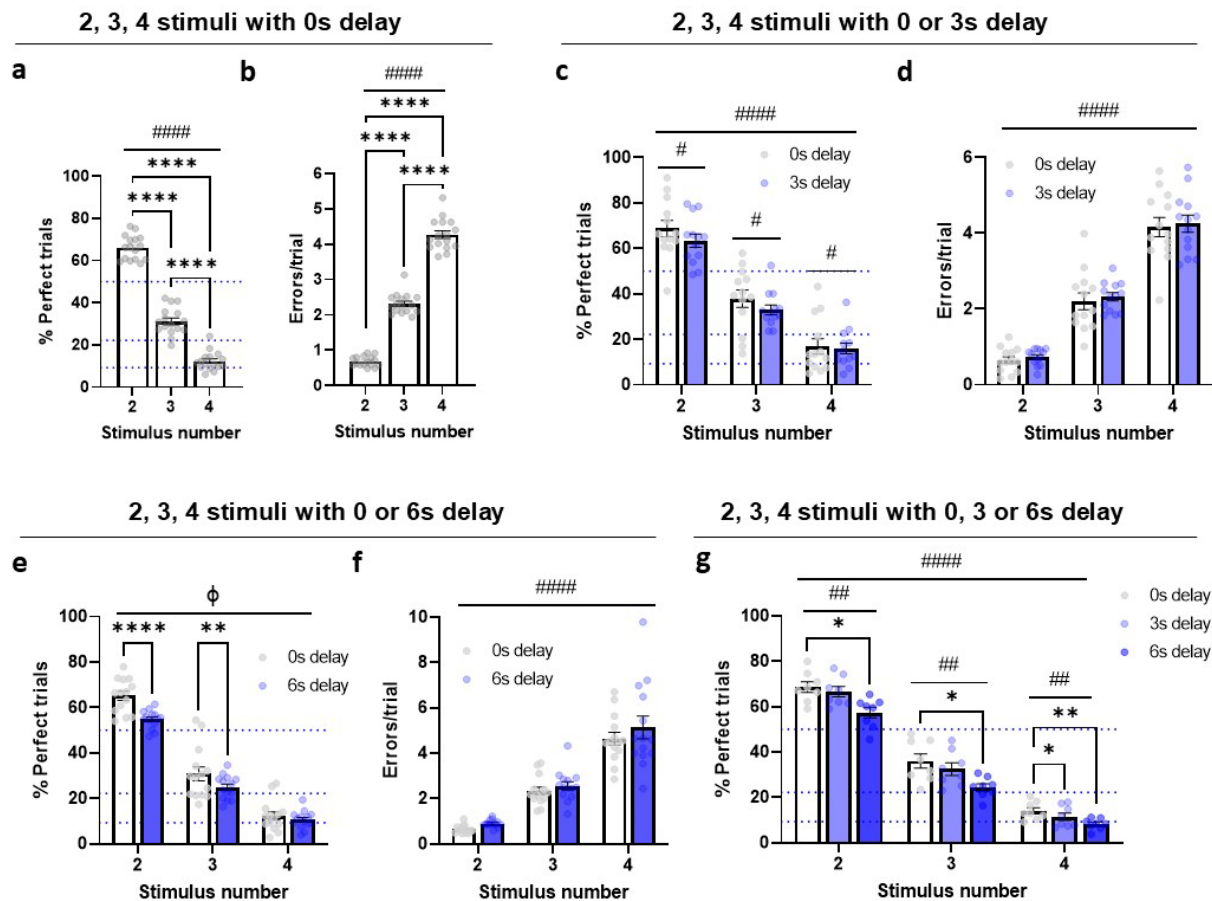


**Figure 18.** Increasing memory load and difficulty parameters negatively impacts rSOWM performance in mice. **a)** Mean rSOWM performance ( $n=14$ ), measured as % perfect trials, on sessions with 2, 3 and 4-stimuli trials. All trials have delay set to 0s. Average shows performance across 49 sessions. **b)** Same as a, but data shown as number of errors/trial. **c)** Mean performance ( $n=14$ ), measured as % perfect trials, on sessions with trials showing 2, 3 and 4-stimuli paired with 0 or 3s delay. Average shows performance across 6 sessions. **d)** Same as c, but data shown as number of errors/trial. **e)** Mean performance ( $n=14$ ), measured as % perfect trials, on sessions with trials showing 2, 3 and 4-stimuli paired with 0 or 6s delay. Average shows performance across 16 sessions.  $\phi$  indicates significant stimulus number x delay interaction. Post-hoc comparisons revealed that delay was significant in the 2 and 3-stimuli but not 4-stimuli trials (e). **f)** Same as c, but data shown as number of errors/trial. **g)** Comparison of rSOWM mean

performance across the nine possible trial type combinations of the three stimulus number and three delay parameter options. Note: no sessions with 0, 3 and 6s delays were given. This graph combines performance on six sessions with 0 or 3s delays (c) and six sessions with 0 or 6s delays (e) to provide with a better visual comparison of the effect of delay across each stimulus number condition. # indicate main effects of stimulus number or delay. \*  $p < 0.05$  Sidak-corrected post-hoc comparisons. Discontinued horizontal lines indicate chance performance on % perfect trials corresponding to each trial type: 50% for 2-stimuli, 22.22% for 3-stimuli and 9.373% for 4-stimuli trials.

Mice were transitioned to the final stage of rSOWM task including 2- and 3-stimulus trials at 0s, 3s or 6s pre-stimulus delays. As observed in previous sessions with 2, 3 and 4 stimuli trials and earlier task stages, rSOWM performance remained sensitive to all task parameters that increase task difficulty: stimulus number, delay and stimulus separation (Figure 19). Both delay and separation significantly impacted rSOWM performance measures in both 2-stimuli (main effect of delay on % perfect trials:  $F(2, 26) = 30.11$ ,  $p < 0.0001$ ; errors/trial:  $F(2, 26) = 16.33$ ,  $p < 0.0001$ ; and separation on % perfect trials:  $F(1, 13) = 16.50$ ,  $p = 0.0013$ ; errors/trial:  $F(1, 13) = 24.30$ ,  $p = 0.0003$ ) and 3-stimuli (main effect of delay on % perfect trials:  $F(2, 26) = 2.389$ ,  $p = 0.1115$ ; errors/trial:  $F(2, 26) = 10.22$ ,  $p = 0.0005$ ; and separation on % perfect trials:  $F(1, 13) = 26.60$ ,  $p = 0.0002$ ; errors/trial:  $F(1, 13) = 23.75$ ,  $p = 0.0003$ ) trials (Figure 19 a,b,d,e). 2-way repeated measures ANOVAs revealed, however, a significant stimuli separation  $\times$  delay interaction affecting % perfect measure on trials with 2 ( $F(2, 26) = 4.255$ ,  $p = 0.0252$ ) and both % perfect and errors/trial measures in trials with 3 stimuli (% perfect trials:  $F(2, 26) = 6.516$ ,  $p = 0.0051$ ; errors/trial:  $F(2, 26) = 3.441$ ,  $p = 0.0472$ ). Post-hoc Sidak-corrected comparisons indicated that separation had significant effect on 0s- ( $p < 0.01$ ) and 3s- ( $p < 0.05$ ) but not in 6s-delay trials. Performance was significantly above chance levels at all delay conditions ( $p < 0.05$ ) except for 3-stimuli trials with 6s delays ( $p = 0.5690$ ). Difficulty to perform above chance on the most difficult trials likely explains the lack of delay effects on the hardest parameters.

We further investigated the impact of difficulty parameters on perseverative errors. Increase in separation (2-stimuli trials:  $F(1, 13) = 4.387$ ,  $p = 0.0564$ ; 3-stimuli trials:  $F(1, 13) = 8.715$ ,  $p = 0.0112$ ) but not delay (2-stimuli trials:  $F(2, 26) = 0.5836$ ,  $p = 0.5650$ ; 3-stimuli trials:  $F(2, 26) = 0.7175$ ,  $p = 0.4974$ ) negatively impacted on the number of perseverative errors (Figure 19 f,g). Together, these data showed that % perfect and number of errors/trial, but not perseverative errors, are measures affected by delay.

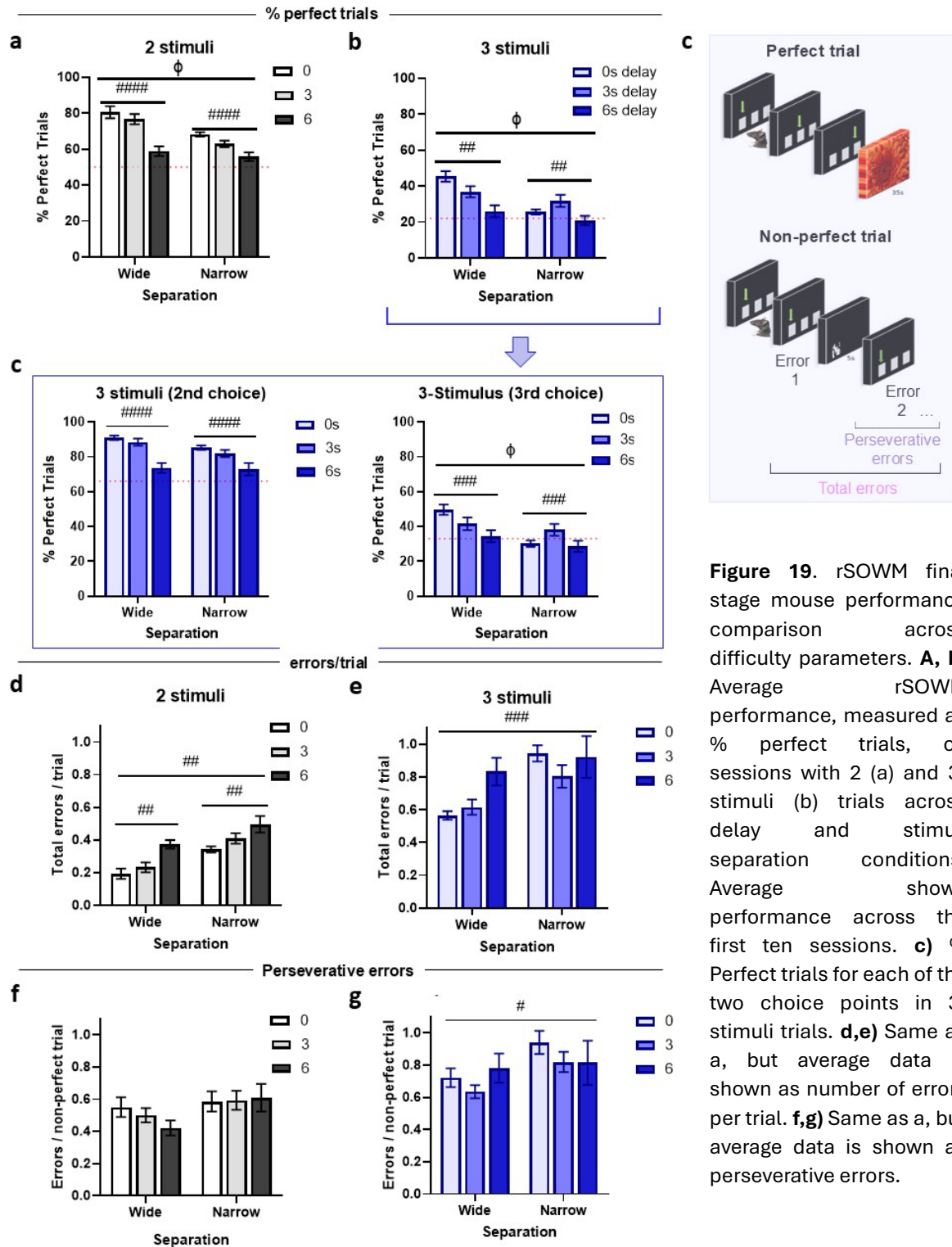


**Figure 19.** rSOWM final stage mouse performance comparison across difficulty parameters. **A, b)** Average rSOWM performance, measured as % perfect trials, on sessions with 2 (a) and 3-stimuli (b) trials across delay and stimuli separation conditions. Average shows performance across the first ten sessions. **c)** % Perfect trials for each of the two choice points in 3-stimuli trials. **d,e)** Same as a, but average data is shown as number of errors per trial. **f,g)** Same as a, but average data is shown as perseverative errors.

Moreover, the probability of being correct on a 3-stimuli trial decreases after each choice. In a given trial, after the first touch, there is a 66.66% probability of making a correct second touch and a 33.33% probability of making a correct third touch. Our prediction was that performance on the second touch of a 3-stimuli trial would be similar or better than in trials with 2-stimuli and that most of the errors would occur on the third stimulus touch. As predicted, % perfect trial performance was significantly above chance levels on the second touch across all delays and separation parameters ( $p < 0.01$ ), but only above chance on the third touch when the delay was 0s (0s  $p = 0.0003$ , 3s  $p = 0.7515$ , 6s  $p = 0.4981$ ) (Fig. *sowm3c*). Both delay and separation affected performance on the second touch (delay main effects:  $F(2, 26) = 27.50$ ,  $p < 0.0001$ ; separation main effects:  $F(1, 13) = 4.188$ ,  $p = 0.0615$ ). There was also a significant separation  $\times$  delay interaction affecting performance on third touches ( $F(2, 26) = 4.758$ ,  $p = 0.0173$ ). Post-hoc comparisons indicated that separation had significant impact on trials with no delay ( $p < 0.05$ ) but not on trials with 3 or 6s delays ( $p = 0.7515$  and  $p = 0.4981$ , respectively). Overall, within trial analysis of 3-stimuli trials confirmed that rSOWM performance is memory load dependent (e.g., error rate increased with stimuli number and delay) and that difficulty increases with each stimulus touch.

rSOWM is a self-ordered task and one of our goals was to investigate whether animals are using or preferring certain sequence patterns to help improve performance. Our hypothesis was that animals would use preferred sequence strategies particularly on trials having higher difficulty (e.g., more stimuli, less separation, greater delays). As observed in Figure 20, mice displayed preferred orders in the wide separation condition but not in trials with narrow separation. Mice predominantly showed intra-individual preferences for either “left-right” or “right-left” sequences in 2-stimuli trials (Figure 20a) and “center-right-left” in 3-stimuli trials (Figure 20e). We quantified their sequence preferences by looking at the entropy of their choice sequences. Entropy in the more difficult narrow separation was close to 1 and significantly higher than in the wider separation (2-stimuli trials:  $p = 0.0016$ ; 3-stimuli trials:  $F(1, 13) = 42.88$ ,  $p < 0.0001$ ), indicating that sequences used were more random and that the first choice in a trial could not predict their subsequent touches (Figure 20b, f). While the stimulus separation disrupted their sequence preferences, sequence entropy was insensitive to delay length (2-stimuli trials:  $F(2, 26) = 0.6891$ ,  $p = 0.5110$ ; 3-stimuli trials:  $F(2, 26) = 0.3269$ ,  $p = 0.7241$ ). It is important to note that, rSOWM performance was significantly above chance across both separation conditions (Figure 20c,g). Thus, although we find clear evidence that mice adopt sequencing strategies when performing the SOWM task, contrary to our hypothesis, strategy use appears to be more prevalent at the easiest task parameters (e.g.,

2 stimuli, wide separation). Moreover, we demonstrate that animals can successfully perform the task above chance levels, despite no obvious sequencing strategy.

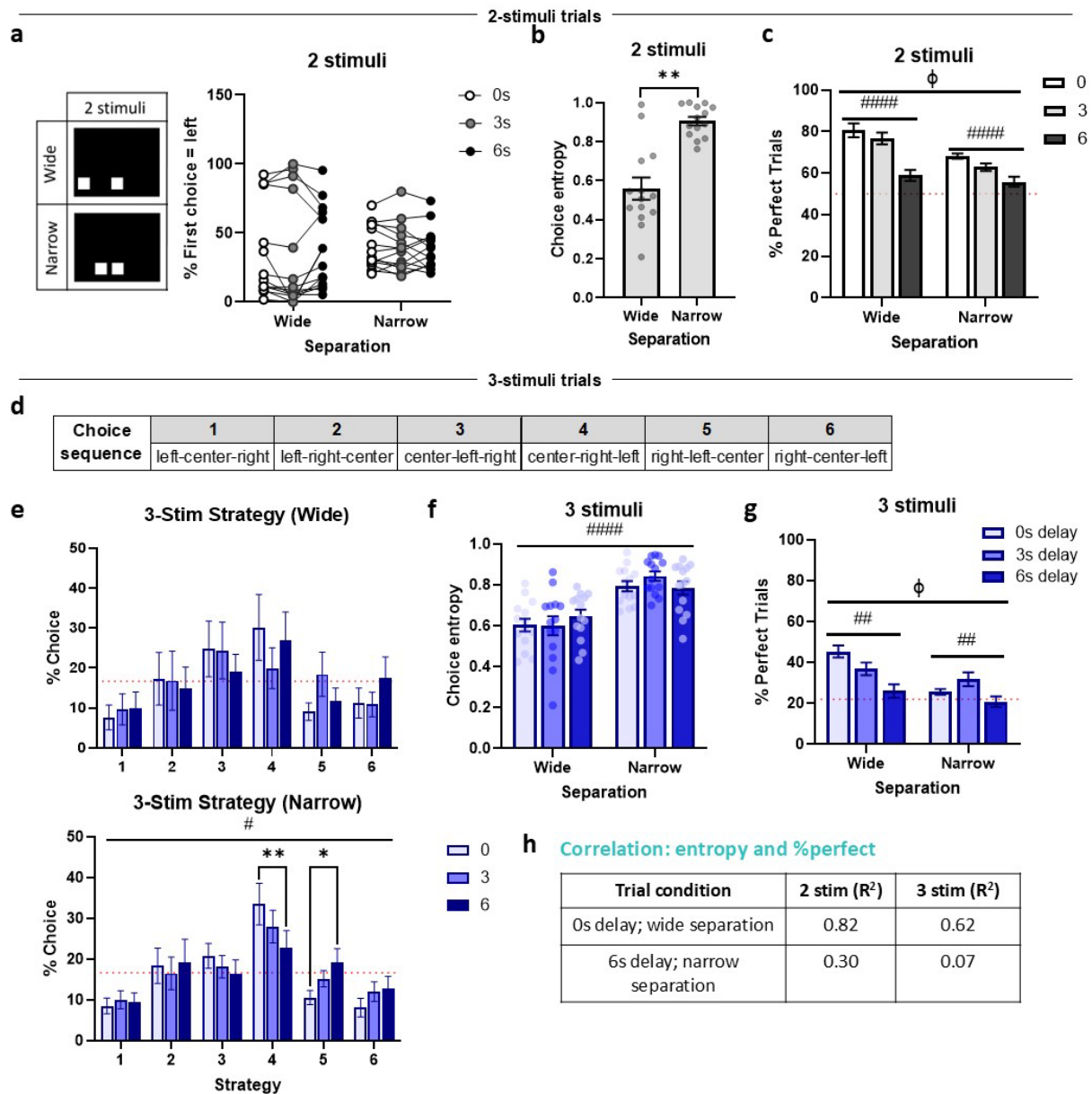


**Figure 20.** Sequence preference and entropy analysis of rSOWM choices. **a)** Representation of the difference between the “narrow” and “wide” separation trial types (left). Percentage of 2-stimuli perfect trials in which the first choice was left across separation and delay conditions (right). **b)** Shannon entropy of sequence choice in 2-stimuli trials across wide and narrow stimuli separation. **c)** %perfect in 2-stimuli trials was significantly above chance across delay and separation parameters. **d)** Table indicating all six possible orders in which 3-stimuli trials can be resolved.

**e)** Choice probability for each of the possible sequence orders mice can use to solve 3-stimuli trials represented across both delay and stimuli separation (wide at the top and narrow at the bottom). **f)** Shannon entropy of sequence choice in 3-stimuli trials across wide and narrow stimuli separation. **g)** %perfect in 3-stimuli trials was significantly above chance across delay and separation parameters. **h)** Correlation analysis between entropy and % perfect trials across trial parameters stimuli number, delay and separation.  $\phi$  indicate significant delay x stimuli separation interaction. # indicate main effects. \* $p < 0.05$ .

### 5.2.2. mPFC 5-HT and DA modulation during rSOWM.

To date, dopamine release in the mPFC has been recorded primarily with indirect methods (e.g., voltammetry) and/or at relatively slow timescales (e.g., microdialysis) in animals performing WM or related paradigms (159, 160). Moreover, few if any studies have examined serotonin (5-HT) dynamics in real time during WM task performance.

To investigate the dynamics of prefrontal DA and 5-HT during WM at a higher temporal resolution, we imaged the D1 receptor-based green, fluorescent GRAB DA indicator gDA3h (161) or the 5-HT4 receptor-based green fluorescent GRAB 5HT indicator g5HT2h (162) in the mPFC of freely moving mice performing the rSOWM paradigm using fiber photometry. 90-minute sessions with 2 or 3 stimuli number and 0 or 6 delay across different stimuli separations were used for fiber photometry experiments.

*In vivo* fiber photometry recordings of mice injected with either GRAB DA3h or GRAB 5-HT2h in the mPFC indicated that DA and 5-HT levels in mPFC were inversely modulated during the different trial events in the rSOWM paradigm (Figure 21). As observed in Figure 21f-g, fluorescent signals indicating release of DA and 5-HT in mPFC showed opposing patterns for each of the two neurotransmitters.

#### mPFC 5-HT and DA modulation at stimulus touch onset

On trial initiation, gDA3h showed a decrease in fluorescent signal with respect to baseline, reaching its lowest peak amplitude at touch onset. This decrease in DA signal was much more pronounced when the touch was correct compared to signal levels at incorrect touch onset (Figure 21f, 22c,e,g,j). gDA3h fluorescent signals during the six seconds prior to stimulus touch responses also showed different profiles between correct and incorrect touches. gDA3h signal was increased prior to correct touches but not different from baseline before incorrect touches (Figure 22a,h).

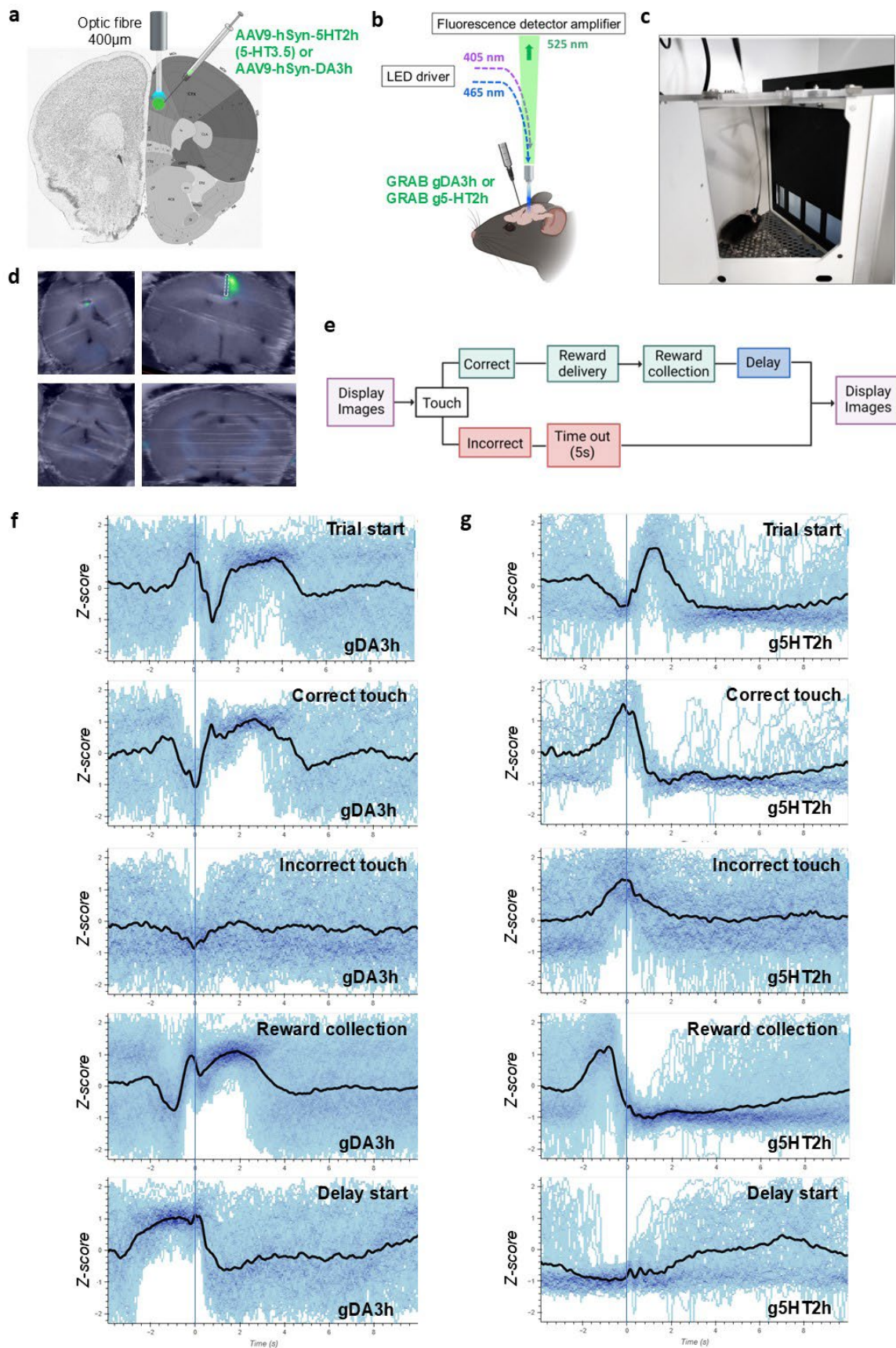


**Figure 21.** DA and 5-HT are distinctly modulated in mPFC during rSOWM. In vivo fiber photometry recordings of mice injected with AAV9-hSyn-5HT2h or AAV9-hSyn-DA3h in medial prefrontal cortex (mPFC). **a)** Surgical strategy prior to fiber photometry recordings. Schematic diagram of a coronal section of a mouse brain indicating the location where the virus was expressed (in green) and the optic fiber placement in PFC (prelimbic). Image modified from: Allen Reference Atlas – Mouse Brain. Available from [atlas.brain-map.org](http://atlas.brain-map.org). **b)** LED and fluorescent detector light wavelengths set-up to monitor DA and 5-HT levels in mPFC. **c)** Picture of a mouse inside the touchscreen chamber connected to the optic fiber patch cord. 5-hole mask is used for rSOWM. **d)** Targeted expression of GRAB 5-HT (5ht3.0) and placement of the optical fiber. Pictures correspond to horizontal (left) and coronal (right) sections of a mouse brain injected with AAV9-hSyn-5HT2h in mPFC obtained by Cryo-fluorescence tomography. **e)** Diagram of the main rSOWM trial events around each choice. After the trial is initiated via a magazine nose-poke, images are displayed on the screen and the shown loop diagram repeats until all the white squares have been touched. The trial then ends and a 30s ITI starts before the next trial. **f)** Fluorescence signal (z-scored  $df/f$ ) detected during rSOWM of two mice injected with AAV9-hSyn-DA3h in mPFC. Graphs show z-score traces of each individual event in blue and the mean z-score values in black. Fluorescence traces are aligned to trial event onset (0s). Data shown for each GRAB sensor is the average of two mice across two sessions of 90 minutes. **g)** Same as f, but mice were injected with AAV9-hSyn-5HT3.0.

Intriguingly, an opposing pattern was observed for 5-HT. 5HT2h fluorescent signal decreased when mice nose-poked to initiate a trial and began increasing 1s before the stimulus touch was made (Figure 21g, 22b,d,f,g,i). Unlike gDA3h, g5HT2h fluorescence increased at stimulus touch onset and did not show significantly different levels prior to correct or incorrect touches. Taken together, these data suggest that DA levels, but not 5-HT levels, in the prelimbic subregion of mPFC during the delay may be predictive of subsequent correct touches.

#### mPFC 5-HT and DA modulation during the delay phase

A monotonic increase in DA release was observed during the first second of the delay period. This increase in gDA3h signal was followed by a rapid dip in fluorescence (Figure 23a, red line, and 23g). We found a significant stimulus number  $\times$  perfect trial interaction ( $F(1, 113) = 6.912, p=0.0098$ ) in the area under the curve (AUC) for the dopamine signal. Subsequent post hoc comparisons revealed that, during the delay period before a correct touch, gDA3h signal was higher in 3stimuli, 6s delay trials relative to 2stimuli, 6s delay trials (Sidak-corrected,  $p=0.0023$ ) as well as relative to 3 stimuli, non-perfect trials (Sidak-corrected,  $p=0.0203$ ). This effect was not observed prior to incorrect touches, suggesting that

increased DA release during the delay period may be involved in bridging the delay when memory conditions are harder (Figure 23c,d).

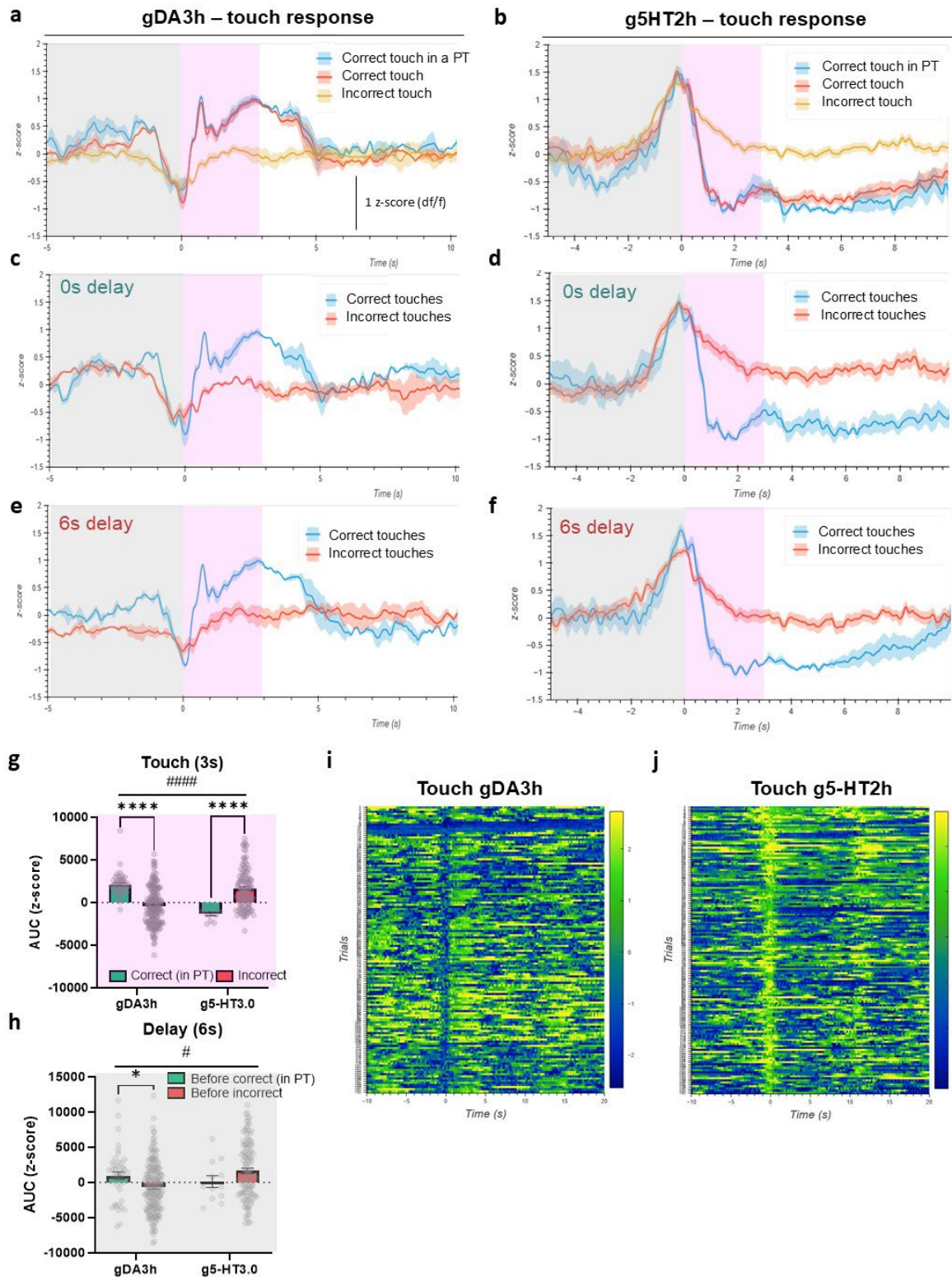


**Figure 22.** Opposite modulation of prefrontal monoamines DA and 5-HT is observed at stimuli touch response. **a, b)** Average z-score of gDA3h (a) and g5-HT2h (b) fluorescent signal aligned to the onset of touch events: correct touch, correct touch in perfect trials and incorrect touch. **c,d)** Average z-score of gDA3h (c) and g5-HT2h (d) fluorescent signal aligned for correct touch onset comparing trials with 0 and 6s delays. **e,f)** Average z-score of gDA3h (e) and g5-HT2h (f) fluorescent signal aligned for incorrect touch onset comparing trials with 0 and 6s delays. **g)** Area under the curve (AUC) comparing gDA3h and g5-HT2h fluorescence (average z-score of  $df/f$ ,  $\pm$  S.E.M) during the 3s following a correct response in perfect trials and an incorrect touch. 2-way ANOVA with repeated measures revealed a significant virus x event interaction ( $F(1, 345) = 55.17, p < 0.0001$ ). **h)** Area under the curve (AUC) comparing gDA3h and g5-HT2h fluorescence (average z-score of  $df/f$ ,  $\pm$  S.E.M) 6s before an incorrect touch or a correct response in a perfect trial and an incorrect touch. 2-way ANOVA with repeated measures revealed a significant virus x event interaction ( $F(1, 345) = 6.024, p = 0.0146$ ). **i)** Heatmap of mean gDA3h fluorescence aligned to all event touches. Heatmap shows fluorescence signal 10s before and 20s after trial event onset. **j)** Heatmap of mean g5-HT2h fluorescence aligned to all event touches. Heatmap shows fluorescence signal 10s before and 20s after trial event onset. Data shown for each GRAB sensor is the average of two mice across two sessions of 90 minutes.

In contrast, 5-HT2h fluorescence slowly and linearly increased along the 6s delay period and reached its peak at the end of the delay when images were displayed again (Figure 23b red line). This rising 5-HT signal is consistent with observations that the rate of firing of dorsal Raphe serotonergic neurons is increased during delays in which animals have to wait for rewards (151). There were no clear differences in g5-HT2h fluorescence between trials with 2 or 3 stimuli number or between perfect and non-perfect trials ( $F(1, 49) = 0.6769, p = 0.4147$  and  $F(1, 49) = 1.576, p = 0.2153$ , respectively) (Figure 23d,f).

#### mPFC 5-HT and DA modulation at reward collection onset

An opposing response to reward collection was also observed between gDA3h and g5-HT2h (2-way ANOVA:  $F(1, 790) = 2558, p < 0.0001$ ). There was a biphasic increase in gDA3h fluorescence and a biphasic decrease in g5HT2h in response to the reward cue and reward collection (Figure 24).

There was a sharp increase in gDA3h fluorescence after a correct touch sharply increased at reward collection onset (Figure 24a,g). The start of this increase in DA release coincided with the sound cue coming from the feeder pump triggered to deliver the reward and peaked at reward collection (Figure 24c). This observed increase in DA release was biphasic. 500ms after the DA increase, DA levels went back to baseline levels and a second slower increase in DA release of similar magnitude begun and lasted a few seconds (Figure 24a). gDA3h signal at reward collection observed after a correct touch in perfect trials

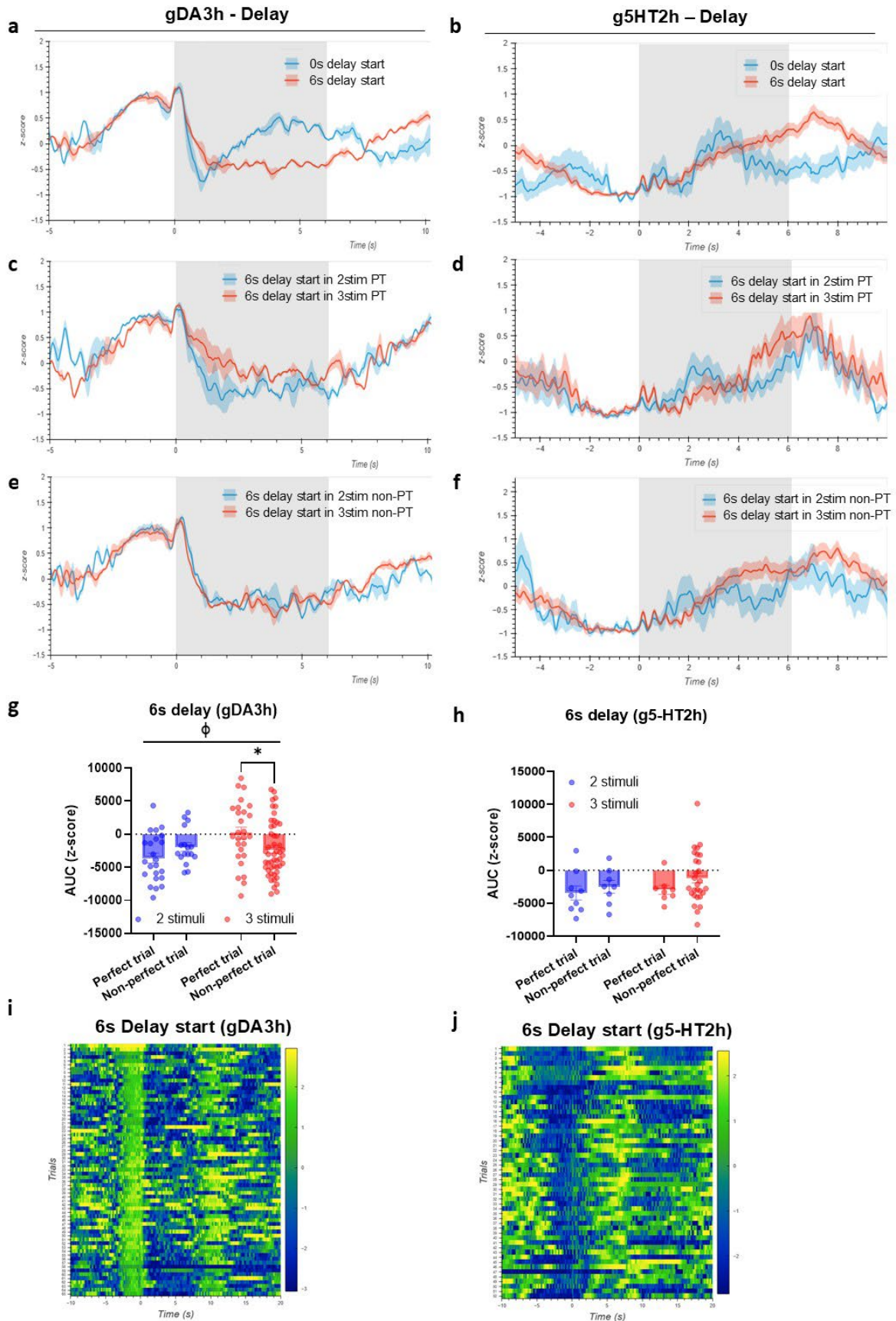


**Figure 23.** Prefrontal DA and 5-HT release dynamics are different during the delay period in rSOWM. **a,b)** Average z-score of gDA3h (a) and g5-HT2h (b) fluorescent signal aligned to delay onset comparing trials with 0 and 6s delays. Delay timer was programmed to start right after reward collection and before images were displayed again and mice could select the next image. **c,d)** Average z-score of gDA3h (c) and g5-HT2h (d) fluorescent signal aligned to delay onset of perfect trials with either 2 or 3 stimulus number. **e,f)** Average z-score of gDA3h (c) and g5-HT2h (d) fluorescent signal aligned to delay onset of non-perfect trials with either 2 or 3 stimulus number. **g, j)** Heatmap of mean gDA3h (g) and g5-HT2h (h) fluorescence (z-score of  $df/f$ ) aligned to delay onset in trials with 6s delays. Heatmaps show fluorescence signal 10s before and 20s after trial event onset. Data shown for each GRAB sensor is the average of two mice across two sessions of 90 minutes.

was not different from the signal observed after a correct touch in non-perfect trials ( $F(1, 790) = 0.004099$ ,  $p=0.9490$ ) (Figure 24e). These results were consistently observed, across stimuli number, separation and delay parameters (Figure 24f).

In contrast, after a sharp 5-HT release increase at correct touch onset, a second small rise occurred immediately after in response to the feeder pump sound cue (Figure 24d). g5-HT2h signal then dropped lower than baseline in a biphasic manner (Figure 24b,d,h), showing a small inflexion in fluorescence right at reward collection onset (see black arrow in Figure 24b). This pattern was observed across all trial conditions. Immediately after reward collection, g5-HT signal continued to decrease reaching the lowest point ~1s after reward collection.

In summary, these results suggest that prefrontal monoamines, 5-HT and DA, are distinctly modulated in the prelimbic region of mPFC during rSOWM. Fiber photometry imaging using fluorescent GRAB sensors indicated that both DA and 5-HT are inversely modulated during stimulus touch (choice) and are both elevated, to differing degrees, in response to reward delivery and collection. DA release appeared to increase briefly during the first second of the delay period, but then decay and remain at low levels for the remainder of the delay. Importantly, average DA levels during the delay period were significantly higher before correct touches compared to incorrect touches, specifically on more difficult trial parameters (e.g., 3 stimuli, 6-s delay). This is the first data, to our knowledge, that shows that DA release dynamics in mPFC during the delay are predictive of subsequent choice outcome in a WM task. In contrast, a slow and monotonic increase in 5-HT release was observed along the delay period, reaching its maximum signal at delay offset, but was not associated with rSOWM task performance.

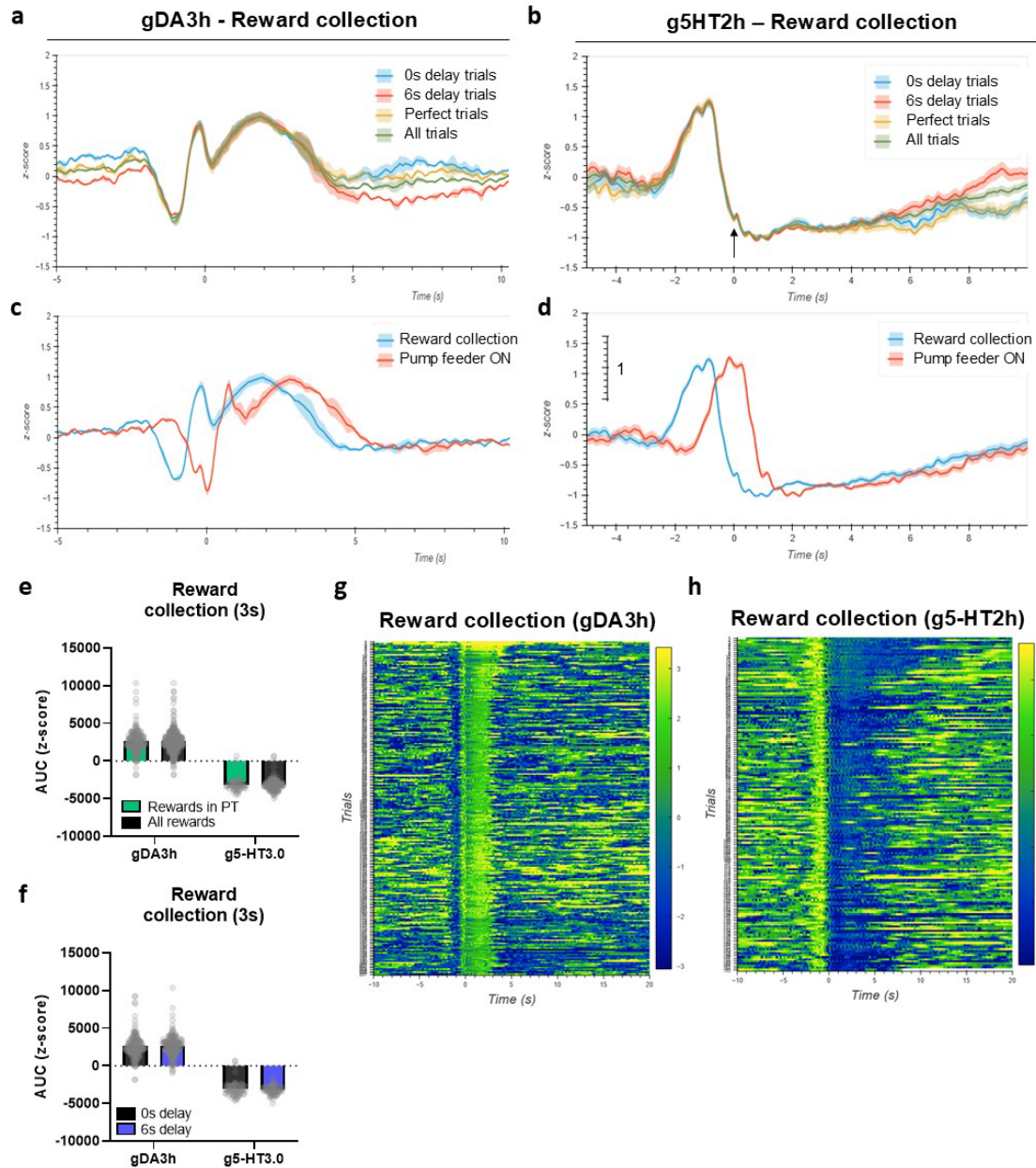


**Figure 24.** Prefrontal DA and 5-HT release modulation in response to reward delivery and collection in rSOWM. **a,b)** Average z-score of gDA3h (a) and g5-HT2h (b) fluorescent signal aligned to the magazine nose-pokes at reward collection after a correct response. Graph compares fluorescence signal at reward collection across different trial conditions: perfect trials, all trials, 0s delay and 6s delay trials. **c,d)** Average z-score of gDA3h (c) and g5-HT2h (d) fluorescent signal aligned to feeder pump sound cue onset (red) and reward collection (magazine nose-poke in blue). **e)** Area under the curve (AUC) comparing gDA3h and g5-HT2h fluorescence (average z-score of df/f,  $\pm$  S.E.M) during the 3s following reward collection in perfect trials vs. all reward collections. gDA3h and g5-HT2h fluorescence were significantly different and in the opposite directions (2-way ANOVA with repeated measures revealed  $F(1, 790) =$

2558,  $p < 0.0001$ ). **f**) Same as e, but comparing fluorescence at reward collection between trials with 0 and 6s delays. gDA3h and g5-HT2h fluorescence were significantly different and in the opposite directions (2-way ANOVA with repeated measures revealed  $F(1, 543) = 2317$ ,  $p < 0.0001$ ). **g, j**) Heatmap of mean gDA3h (g) and g5-HT2h (h) fluorescence aligned to reward collection. Heatmaps show fluorescence signal 10s before and 20s after trial event onset. Data shown for each GRAB sensor is the average of two mice across two sessions of 90 minutes.

### **5.3. CHAPTER 3. The rodent Delay and Reward Discounting (rDRD) paradigm in mice.**

#### **5.3.1. rDRD task validation and performance measures in mice.**

In this study, we have developed and validated a second novel touchscreen paradigm, the Delay and Reward Discounting (DRD) paradigm for rodents. On each DRD trial, two visual stimuli are presented side-by-side on a touch screen. Animals are then freely allowed to touch one and, after a programmed delay period, a liquid reward is delivered. Reward magnitude and delay on a given trial are uniquely dependent on the chosen stimulus. Each combination of reward size (15, 25, 35, 50 $\mu$ L) and delay (0, 2, 4 or 6s) are represented by the visual properties of 16 different images. Any systematic biases can be corrected by titrating forced choice trials.

Mice were first trained on the 0 second delay condition (4 images total – see Methods). Criteria to move to the next stage was to perform at  $\geq 80\%$  correct (selecting the higher reward option) across two consecutive sessions. Next, trials with 12 second delays were added to the session (8 images total – see Methods). Mice were maintained at this stage until performance was stable across 3-4 sessions.

Once mice reached the final stage of the task and were presented with paired combinations of 16 images. Preference analysis for each trial type was performed: “Same Delay”, “Same Reward”, “Forced Choice” and “Free Choice” (Figure 25). As expected, choice data on trials with “Same Delay” showed that mice preferred larger rewards particularly when the size ratio between the two choices is high ( $F(5, 35) = 32.55$ ,  $p < 0.0001$ ) (Figure 25a). Choice data on trials with “Same Reward” also indicated that mice preferred larger rewards particularly when the size ratio between the two choices is high ( $F(5, 35) = 35.21$ ,  $p < 0.0001$ ) (Figure 25b).

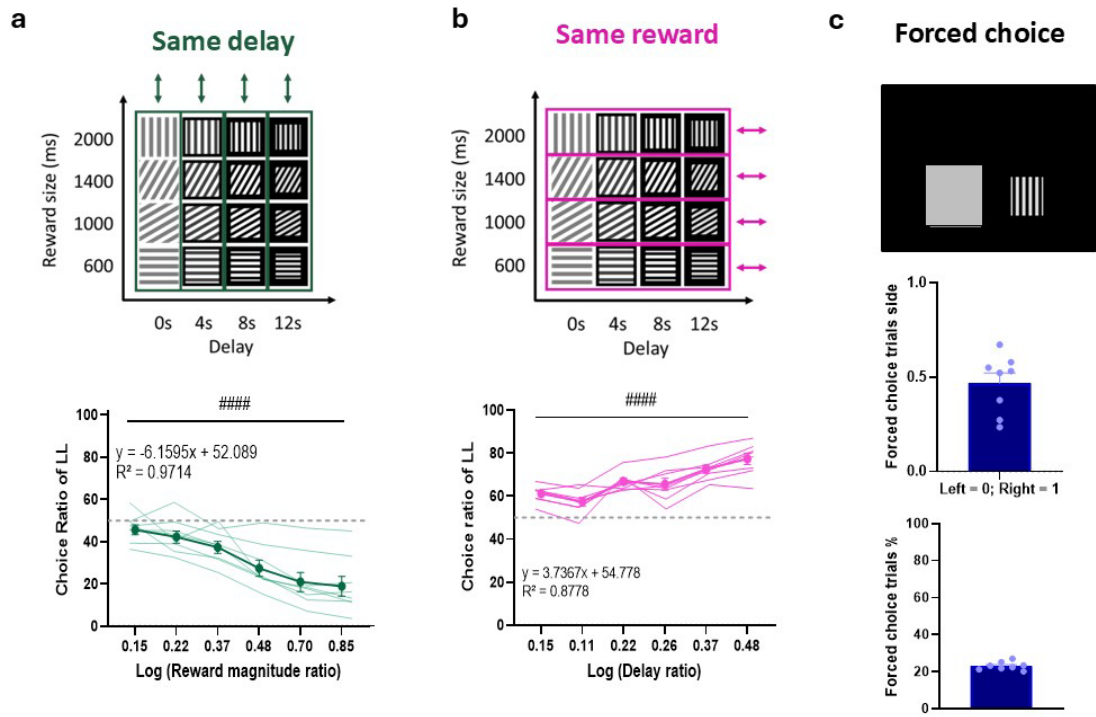


**Figure 25.** Choice preference for reward and delay magnitudes on “Same Delay” and “Same Reward” trials in rDRD. **a)** Schematic representation of the possible pairing groups in trials with the same delay for both images (top). Image pairings on each given trial were made within each of the four columns. Choice ratio of the small reward option across the six possible reward magnitude ratios (bottom). **b)** Schematic representation of the possible stimulus pairing groups in trials with the same reward magnitude in both options (top). Choice ratio of the short delay option across the six possible delay magnitude ratios (bottom).

Mice were given forced choice trials to help prevent side bias (Figure 25c). When mice responded to the image on the same screen location three consecutive times, the next trial was set to be a “forced choice” trial. These trials presented the choice between one of the 16 images and a grey square image that was inactive to the touch. The grey square stimulus was presented on the same side of the screen where the last three choices were made, and mice were forced to touch the image opposite from the grey square in order to obtain a reward and move on with the session. “Forced choice” trials were given to ensure that mice selected each of the 16 stimulus and were exposed to the delay and reward sizes associated with them; as well as to avoid acquisition of potential side bias. On average, mice received 23.11% of forced choice trials per session and did not show side bias (One sample t-test  $p=0.5764$ ) (Figure 25c bottom).

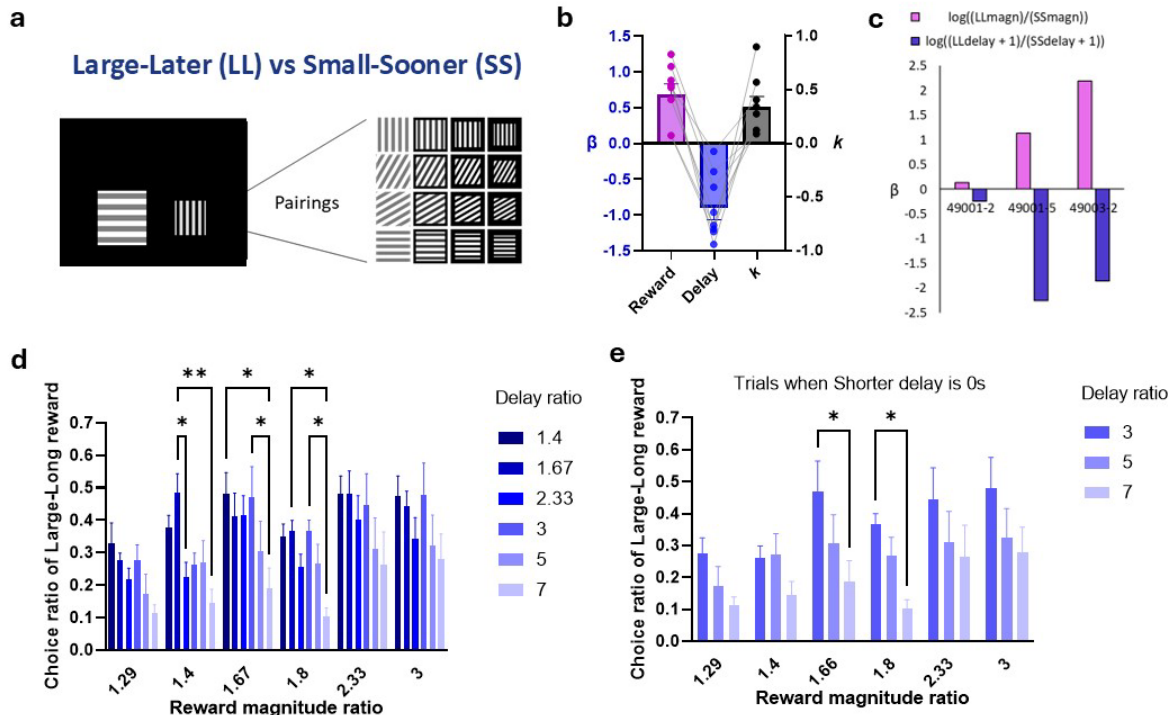


**Figure 26.** Discounting rate and sensitivity to delay and reward magnitude in rDRD "Free Choice" data. **a)** Example pairing in a "Free Choice" trial. **b)** Logistic regression model of choice data showing average discounting rate ( $k$ ) and sensitivity to reward magnitude ( $\beta_{\text{reward}}$ ) and delay ( $\beta_{\text{delay}}$ ). **c)** Choice ratio of the Large-Long (LL) reward option across all "Free Choice" stimuli combinations. **d)** Same as c, but analysis is limited to trials with 0s as the Small-Short (SS) stimulus delay. Data shown as MEAN+/-S.E.M.

Multilevel logistic regression modeling is applied to the choice data on "free choice" trials to estimate the separate parametric contributions of sensitivity to reward magnitude ratio and relative delay. Mice displayed preferences for higher reward ( $\beta_{\text{reward}}$ : mean=0.69, +/-S.E.M=0.14) and shorter delay ( $\beta_{\text{delay}}$ : mean=0.90, +/-S.E.M=0.16) with a higher sensitivity for delay ( $\beta_{\text{delay}}$ ). We observed that although mice show an overall higher mean discounting based on delay length, there was high heterogeneity in reward and delay discounting rates. The hyperbolic parameter in the logistic regression model applied to the trials when the shorter delay was 0 indicated high discounting rates (mean  $k=0.34$ ) (Figure 26b).

### 5.3.2. mPFC DA and 5-HT modulation during rDRD.

*In vivo* fiber photometry recordings of mice injected with AAV9-hSyn-5HT2h (5-HT3.5) or AAV9-hSyn-DA3h in the mPFC indicated that DA and 5-HT release dynamics in mPFC are different in response to the different phases and trial events that occur in rDRD trials.

Similar to our fiber photometry data in rSOWM, prefrontal DA and 5-HT were modulated in the opposite manner during the stimulus touch (choice) and reward collection phases of the rDRD task (Figure 27). While gDA3h fluorescence decayed as the mouse approached the screen to choose a stimulus, g5-HT2h fluorescence showed the opposite pattern (Figure 27e-f). Prefrontal DA release increased in response to the feeder sound cue and peaked at reward collection onset (Figure 27g). 5-HT release, however, decayed lower than baseline after stimulus touch and showed a small transitional increase in fluorescence at reward collection onset (Figure 27h), in the same manner as we had previously observed in rSOWM.

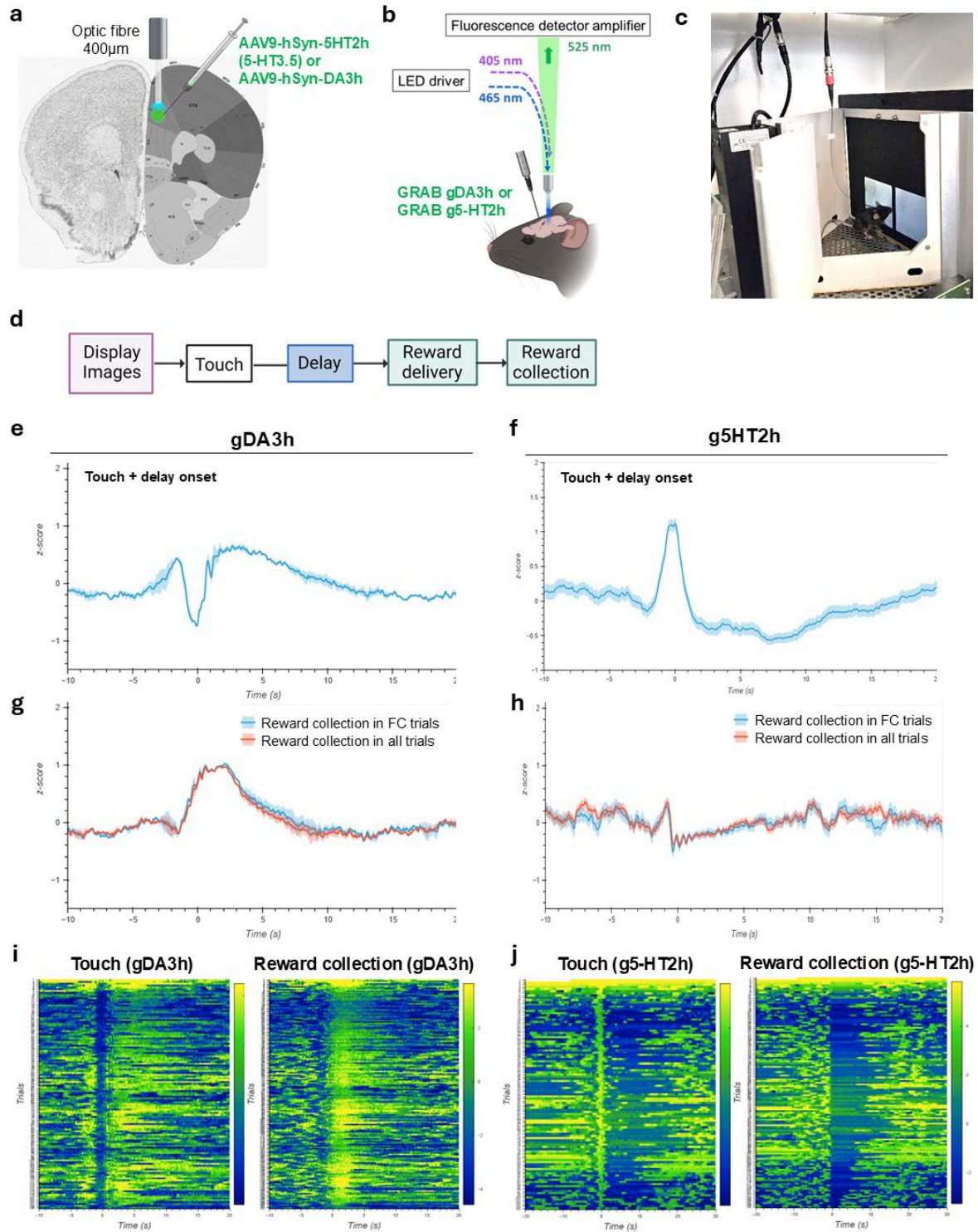


**Figure 27.** Prefrontal DA and 5-HT are distinctly modulated in mPFC during rDRD. In vivo fiber photometry recordings of mice injected with AAV9-hSyn-5HT2h (g5-HT2h) or AAV9-hSyn-DA3h (gDA3h) in medial prefrontal cortex (mPFC). **a)** Surgical strategy prior to fiber photometry recordings. Schematic diagram of a coronal section of a mouse brain

indicating the location where the virus was expressed (in green) and the optic fiber placement in the prelimbic area of the PFC. Image modified from: Allen Reference Atlas – Mouse Brain. Available from atlas.brain-map.org. **b)** LED and fluorescent detector light wavelengths set-up to monitor DA and 5-HT levels in mPFC. **c)** Picture of a mouse inside the touchscreen chamber connected to the optic fiber patch cord. 2-hole mask is used for rDRD. **d)** Diagram of the main rDRD trial events around each choice. After the trial is initiated via a magazine nose-poke, two images are displayed on the screen. Touching one of the two stimulus triggers reward delivery after a set delay (refer to methods section 4.4.1 for more details). Trial ends with an ITI period. **e)** Fluorescence signal (z-scored  $df/f$ ) detected during rDRD of two mice injected with AAV9-hSyn-DA3h in mPFC. Graphs show z-score traces aligned to the stimulus touch and start of the delay period, which starts when the stimulus is selected. Data shown for each GRAB sensor is the average of two mice across two sessions of 90 minutes. **f)** Same as, but mice were injected with g5HT2h. **g)** gDA3h fluorescence signal (z-scored  $df/f$ ) aligned to the reward collection onset in forced choice (FC) trials compared to reward collection across all trial types. **h)** Same as g, but for mice injected with g5HT2h. **i)** Heatmap of mean gDA3h fluorescence aligned to stimulus touch (left) and reward collection (right). Heatmaps show fluorescence signal 10s before and 20s after trial event onset. **j)** Heatmap of mean g5-HT2h fluorescence aligned to stimulus touch (left) and reward collection (right). Heatmaps show fluorescence signal 10s before and 20s after trial event onset.

To understand whether and how DA and 5-HT release dynamics are associated with choice and processing of different delay and reward magnitude options, we examined gDA3h or g5-HT2h fluorescence signals in the prelimbic region of mPFC while mice performed the rDRD task. gDA3h or g5-HT2h fluorescence signals during the 6s prior to stimulus choice were not significantly different depending on the delay (gDA3h:  $F(3, 293) = 0.2982$ ,  $p=0.8267$ ; g5-HT2h:  $F(3, 211) = 0.6922$ ,  $p=0.5577$ ) (Figure 28i,j) or reward magnitude chosen (gDA3h:  $F(3, 293) = 0.9252$ ,  $p=0.4289$ ; g5-HT2h:  $F(3, 211) = 1.714$ ,  $p=0.1651$ ) (Figure 28e,f).

Upon stimulus selection (touch) gDA3h fluorescence sharply increased during the first second of the delay period (Figure 28a, 4a). After this initial increase in DA release, gDA3h signal started decaying until the delay period was over and the reward pump triggered. At that point, DA release increased again, peaking at the time of reward collection (Figure 28a,e). The time window during which gDA3h signal remained elevated after reward collection was dependent on both the delay chosen ( $F(3, 355) = 5.375$ ,  $p=0.0013$ ) (Figure 28c,g,i) and the reward magnitude ( $F(3, 355) = 35.89$ ,  $p<0.0001$ ) (Figure 20c,e). The longer the magnitude, the longer the increase in DA release lasted (1-2s difference between each reward size) (Figure 30c).

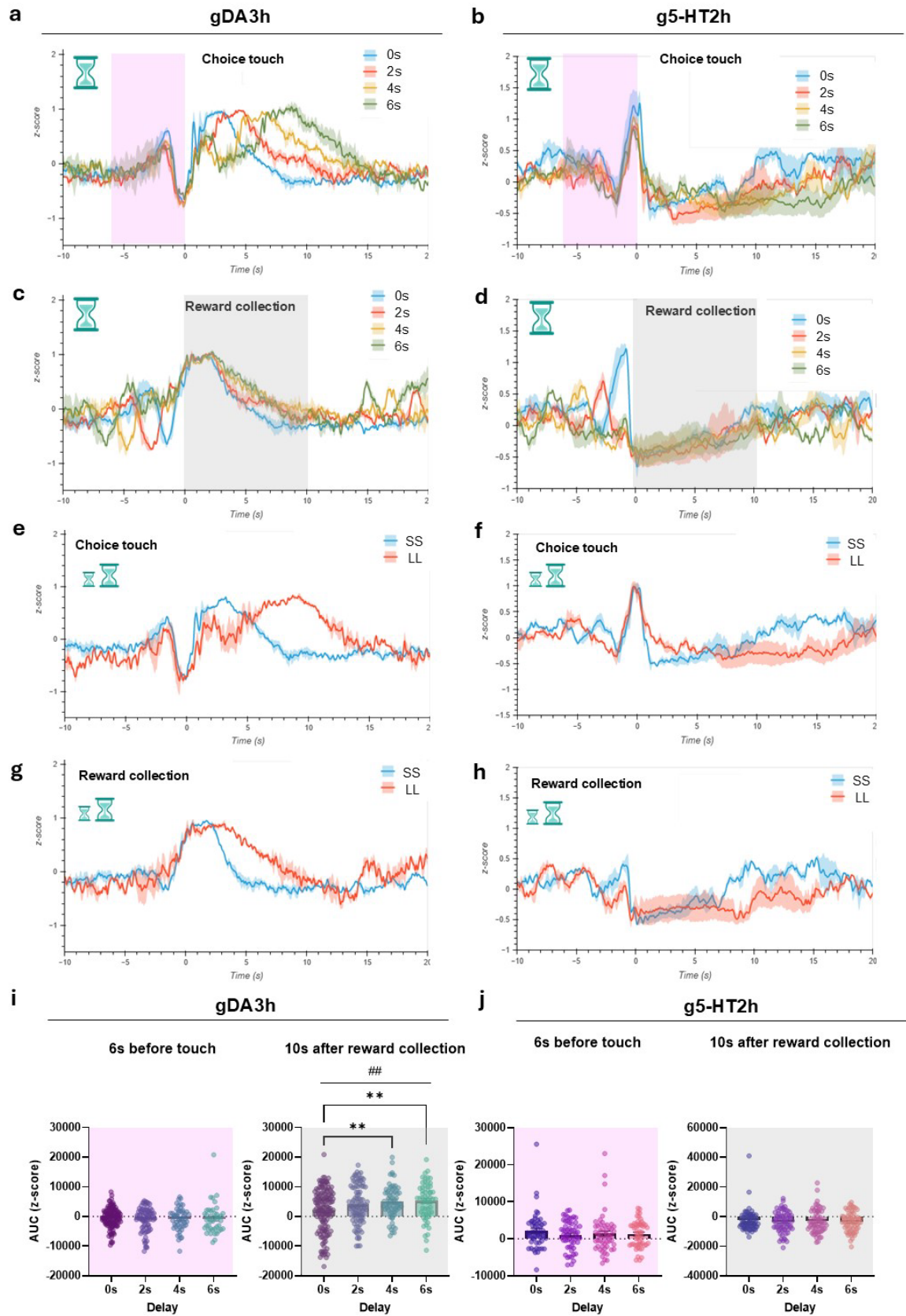


**Figure 28.** Prefrontal DA and 5-HT release dynamics during stimulus choice and reward collection across the four possible delays chosen in rDRD. **a,b)** Average z-score of gDA3h (a) and g5-HT2h (b) fluorescent signal aligned to stimulus touch. Graph compares fluorescence signal for each of the four possible delay conditions. **c,d)** Average z-score of gDA3h (c) and g5-HT2h (d) fluorescent signal aligned to reward collection. **e, f)** Same as a,b, but comparison is between trials in which the shorter-sooner (SS) or longer-larger (LL) stimulus option was chosen. **g,h)** Same as c,d, but comparison is between trials in which the shorter-sooner (SS) or longer-larger (LL) stimulus option was chosen. **i)** Area under the curve (AUC) comparing gDA3h and g5-HT2h fluorescence (average z-score of df/f, +- S.E.M) during the 6s preceding stimulus touch (left) and 10s from reward collection (right) for each of the chosen delay options. **j)** Same as i, but for g5-HT2h GRAB sensor. # indicate main effects \*indicate Sidak-corrected Post-hoc comparisons.

By contrast, g5-HT2h fluorescence showed a biphasic decrease that began immediately after stimulus touch but did not get to the lowest signal values until the reward was collected (Figure 28d,f). g5-HT2h fluorescence remained below baseline for multiple seconds after reward collection. The duration of this negative signal window was dependent on the reward magnitude collected ( $F(3, 259) = 4.530$ ,  $p=0.0041$ ) (Figure 30f). The larger the reward volume, the longer it took g5-HT2h fluorescence to go back up to or slightly higher than baseline (Figure 30d,f); thus scaling to reward magnitude.

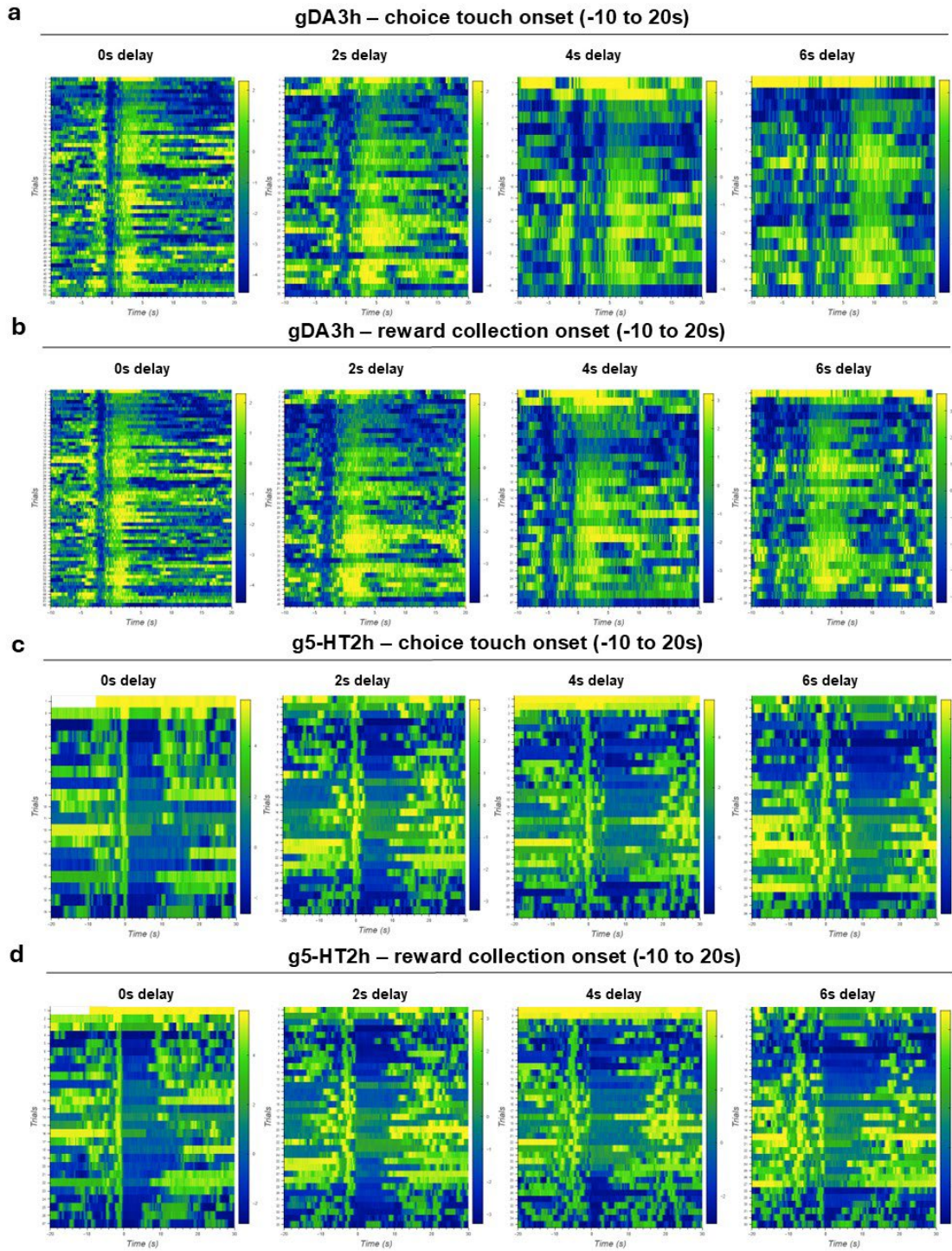


**Figure 29.** Heatmaps representing prefrontal DA and 5-HT release dynamics during stimulus choice and reward collection events in rDRD across the four delay options. All heatmaps include data from 10s before to 20s after the indicated event. **a)** gDA3h fluorescence signal (z-scored  $df/f$ ) is aligned to stimulus touch. **b)** gDA3h fluorescence

signal (z-scored df/f) is aligned to reward collection. **b)** g5-HT2h fluorescence signal (z-scored df/f) is aligned to stimulus touch. **b)** g5-HT2h fluorescence signal (z-scored df/f) is aligned to stimulus touch.

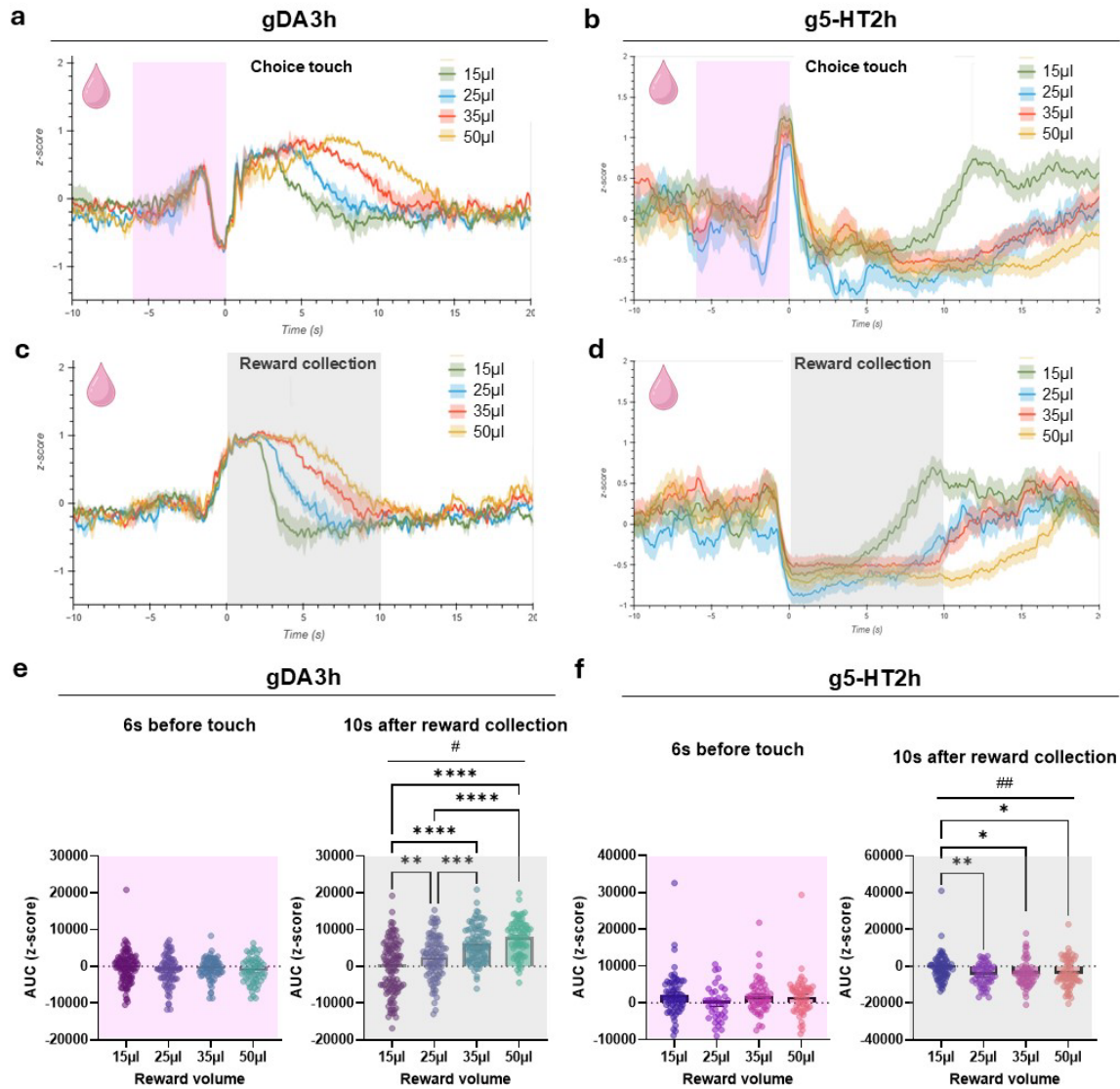


**Figure 30.** Prefrontal DA and 5-HT release dynamics during stimulus choice and reward collection across the four possible reward magnitudes chosen in rDRD. **a,b)** Average z-score of gDA3h (a) and g5-HT2h (b) fluorescent signal aligned to stimulus touch. Graph compares fluorescence signal for each of the four possible reward size conditions. **c,d)** Average z-score of gDA3h (c) and g5-HT2h (d) fluorescent signal aligned to reward collection. **e)** Area under the curve (AUC) comparing gDA3h and g5-HT2h fluorescence (average z-score of df/f,  $\pm$  S.E.M) during the 6s preceding stimulus touch (left) and 10s from reward collection (right) for each of the chosen delay options. **f)** Same as e, but for g5-HT2h GRAB sensor. # indicate main effects \*indicate Sidak-corrected Post-hoc comparisons.

#### 5.4. DA modulation in dorso-lateral striatum (DLS) during rDRD.

Very few studies looking at prefrontal monoamine dynamics during WM and DD with direct or high time-resolution techniques, such as imaging techniques using genetically encoded fluorescent sensors, exist. However, numerous studies have used GRAB<sub>DA</sub> sensors to look at DA modulation in striatum during reward processing (163). In order to validate our methods and compare the DA dynamics between mPFC and DLS during delay and reward processing, we performed the same rDRD fiber photometry experiments in two mice injected with gDA3h sensor in DLS.

Consistent with previous studies looking at reward signal and processing in striatum (163), our data showed a sharp large increase in gDA3h fluorescence at both touch (response to cue) and reward collection (Figure 31). The peak fluorescence size at stimulus touch response was dependent on the delay length ( $F(3, 375) = 15.95$ ,  $p < 0.0001$ ) but not reward magnitude ( $F(3, 375) = 0.3307$ ,  $p = 0.8031$ ) (Figure 31a,b,i).

The fluorescence increase at reward collection onset was dependent on the delay ( $F(3, 397) = 226.0$ ,  $p < 0.0001$ ) and reward magnitude ( $F(3, 397) = 5.931$ ,  $p = 0.0006$ ) of the image chosen. The longer the delay and reward chosen was, the higher observed DA release increase (Figure 31b,d,j). Interestingly, the elevation in DA signal in trials in which the 0s delay option was chosen was near baseline (Figure 31b). This result is consistent with literature suggesting that DA increase responses to reward disappear as the association between cue and immediate reward is learned (e.g., reward prediction error). In these results, we can observe that DA response to reward prediction error disappears when >2s delay between the cue and reward is added.

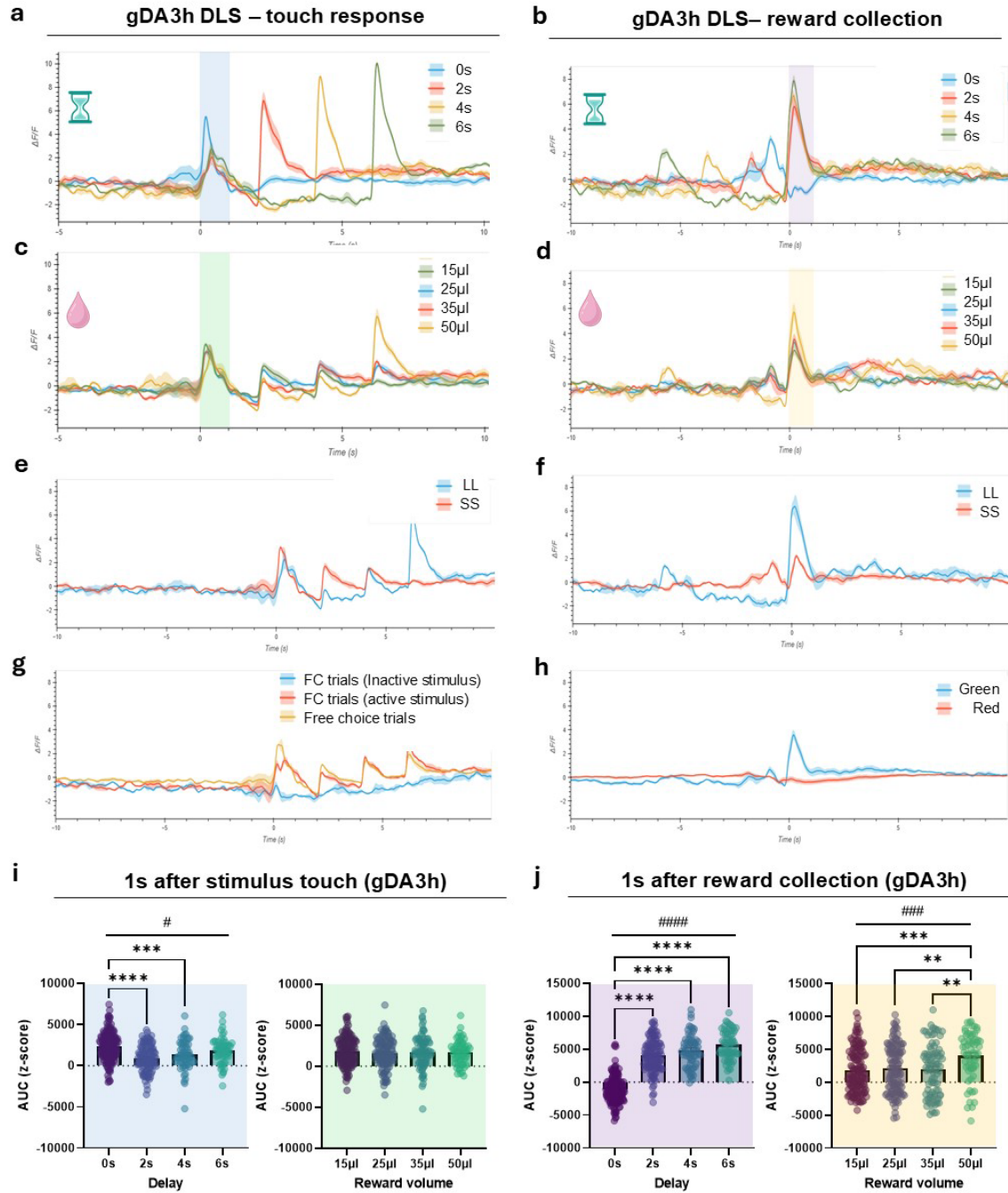


**Figure 31.** DA release dynamics in DLS during rDRD. **a,b)** Average z-score of gDA3h fluorescent signal aligned to stimulus touch (a) and reward collection (b) across the different delay options chosen. **c,d)** Average z-score of gDA3h fluorescent signal aligned to stimulus touch (c) and reward collection (d) across the different reward magnitude options chosen. **e,f)** Comparison of the average z-score of gDA3h fluorescent signal aligned to stimulus touch (e) and reward collection (f) between trials in which the shorter-sooner (SS) or longer-larger (LL) stimulus option was chosen. **g)** Comparison of the average z-score of gDA3h fluorescent signal aligned to stimulus touch in all free-choice trials,

touches to the grey, inactive image of a forced-choice (FC) and touch to the active image of a FC trial. **h)** Average z-score of green DA3h fluorescent signal aligned to reward collection compared to the signal **i)** Area under the curve (AUC) of the gDA3h fluorescent signal (average z-score of  $df/f$ ,  $\pm$  S.E.M) 1s before stimulus touch across the four delay (left) and four reward magnitude (right) options chosen. **j)** Same as i, but for gDA3h fluorescence detected 1s after reward collection.

## 6. Discussion

### 6.1. The rodent Self-Ordered Working Memory paradigm.

During this project, I developed and behaviorally validated two novel psychometric touchscreen tasks to assess working memory and delay discounting in rodents. The first paradigm, the rodent Self-Ordered Working Memory task (rSOWM), was closely modeled after the human SOPT (10) and SWM tasks (11). On each rSOWM trial, two or more visual stimuli are presented within a horizontal array across the touchscreen (presentation phase), animals are then freely allowed to select the stimuli in any desired order (choice phase) and are rewarded each time they select a unique visual stimuli location (reward phase). Returning to select any of the previously visited stimulus locations within a trial, results in error feedback and a timeout period that delays the opportunity for the next reward. Multiple trial configurations, that varied in difficulty, were presented randomly to the animals within each rSOWM session.

rSOWM task difficulty level was increased by three distinct parameters: number of stimuli (2, 3 or 4), spatial location and separation between stimuli (wide, narrow), and delay within-trial stimulus presentation (0 or 6s). Currently, the standard CANTAB SWM task for humans only varies the number of stimuli presented to increase spatial working memory load. Our results suggest that adding delays and systematically varying the spacing between boxes may offer further levels of difficulty for this CANTAB task. rSOWM performance in both rats and mice was sensitive to these common parametric manipulations that impact WM performance in humans. As predicted, rSOWM performance was significantly reduced as task difficulty increased. The capability to parametrically manipulate task difficulty by taxing different key components (memory load, pattern separation, delay length) involved in WM performance is a critical design feature in rSOWM that may be key to helping understand the neurobiology and circuitry behind each component parameter.

To address whether self-sequencing the stimuli choices was important in rSOWM performance, we developed a task variant of the SOWM, the Experimentally-Ordered Working Memory (EOWM) task. This variant constrained the rats' ability to self-sequence their responses by requiring them to initiate each trial from a fixed location on the screen. Interestingly, data with this variant revealed that animals present

higher performance levels when they are allowed to strategically order their choices. Based on this observation, it could be hypothesized that strategic sequencing of touch choices could be beneficial, particularly in trials with highest difficulty (e.g., higher stimuli number). However, when we analyzed measures of entropy and touch sequencing, we found that both rats and mice displayed preferential sequences and engaged in significantly more sequencing strategies when the task parameters were easy (e.g., 2 stimuli trials). This result is highly consistent with bias analyses reported in the 2-choice DNMTF and TUNL paradigms that demonstrate significant use of postural or response mediating strategies (37, 38) (44). Together, these results could indicate that: 1) defaulting to preferred sequences might be only used when WM performance is not sufficiently challenged or even necessary; 2) brain processes required to successfully perform in many of the classic two-choice WM tasks (e.g., DNMTF) could be important to processes involved in “switching” or “defaulting” towards simpler learned motoric strategies when task is easy that, perhaps, are different from those needed during WM.

Although choice self-sequencing increased rSOWM performance, it is important to note that performance on the more difficult conditions of rSOWM (e.g., 3- and 4-stimuli trials in which choice entropy was near 1) as well as performance in EOWM were still significantly above chance levels. These results indicate that, even if rodents express preferred touch sequencing in the two-choice, easier trial conditions, sequencing is not necessary to resolve rSOWM trials. Another important feature of the rSOWM, especially when compared to other WM operant tasks, is that the screen location of each of the visual stimuli is pseudo-randomized so that the stimuli configurations are different even within the same trial type (e.g., different location and separation between stimuli). In other words, two trials with the same exact parameter conditions and even difficulty metrics can have images presented in different locations (e.g., in 2 stimuli trials: one stimulus on each side of the screen, both stimuli are closer to the left, both stimuli are closer to the center, etc.) making each trial much more unpredictable and, therefore, challenging the use of motoric strategic sequences to solve the task. In alignment with these findings, data obtained across the various experimental manipulations conducted in this thesis project (e.g., hippocampal lesions, modafinil administration) revealed significant differences in rSOWM performance selectively in 3-stimuli trials. Together, results in this thesis indicate that increasing the number of stimuli beyond two choice options may be essential to study and detect changes in WM.

We have successfully adapted and translated the rSOWM task for both rats and mice. After our first rSOWM experiments performed in rats, there were multiple reasons why we wanted to adapt the

rSOWM task for mice. First, we wanted these automated tasks to have the flexibility to be used in both rodent species so that key results might be reproduced and generalized across species. This is important for the robustness of our findings as well as for the potential translation to clinical studies. Second, some of the tools and imaging techniques we used to investigate prefrontal DA and 5-HT modulation during task performance have been mostly or entirely validated in mice. Third, genetic mouse models to investigate the role of specific DA or 5-HT receptors in WM are more readily available in mice.

In the present study, we investigated whether the rSOWM paradigm was sensitive to dysfunction in hippocampal CA3 and DG subregions. The hippocampus has been extensively linked with spatial working memory (164, 165) and pattern separation (166). Due to the reciprocal interconnections between DG and CA3, it has been proposed that these two subregions play a role in both processes. However, the CA3 subregion has been more highly linked to maintaining short-term memory representations (164), whereas the DG subregion appears to support pattern separation (166). Pattern separation is the mechanism that allows distinguishing overlapping patterns of neural activity so that they are retrieved separately from each other. It has been proposed that the mechanism by which partially overlapping information is kept separate in hippocampus occurs thanks to the sparse connectivity in the mossy-fibers connecting the DG granular cells and CA3 pyramidal cells, which decreases the probability that similar subsets of DG cells will send similar inputs to the same cells in CA3 (167).

Based on this prior literature, we hypothesized that rats with lesions to hippocampal subregions CA3 or DG might show differential impairments in rSOWM parameters. While we found no group differences in performance on the easier 2-stimuli trial conditions across groups, both CA3 and DG lesioned groups presented differential impairments in 3-stimuli trials. CA3-lesioned animals were significantly impaired only in trials with 6s delay, whereas lesions in DG caused profound performance deficits that were independent of delay. Consistent with Gilbert & Kesner (2006) (168), these results suggested that CA3 may be critical to maintain spatial representations over time and that DG lesions may impair execution and monitoring of a sequence of spatial responses. These results also served to validate the rSOWM as a test of spatial WM paradigm that is sensitive to hippocampal dysfunction.

Together, the results in this chapter indicated that rSOWM is a robust rodent analogue of SWM, providing a powerful translational tool for elucidating the mechanisms of WM and evaluating dysfunction in relevant brain regions.

## 6.2. rSOWM sensitivity to putative cognitive enhancer Modafinil

To further validate rSOWM and its potential as a tool to evaluate drug efficacy and to investigate its neuropharmacological basis, we examined whether rSOWM task performance was sensitive to the effects of putative cognitive enhancing drugs in normal, healthy adult rats. We first assessed task sensitivity to modafinil (2-[(Diphenylmethyl)sulfinyl]acetamide, Provigil 1997), a wake-promoting agent with demonstrable efficacy in the treatment of narcolepsy (169). Although its precise mechanisms of action are unknown, modafinil has been shown to significantly improve performance on tests of executive cognition in non-sleep deprived healthy volunteers (170, 171) and in patients with neuropsychopathological disorders (171, 172). However, the effect of modafinil on spatial WM remains unclear. Although Turner, et al. 2003 (170) reported no significant effects of modafinil on WM in healthy volunteers, a later study demonstrated a significant cognitive enhancing effect, apparent only in a more difficult version of the human CANTAB SWM task (with increased number of boxes) (158).

Mirroring these findings in healthy human volunteers, we demonstrated that low-moderate doses of modafinil (8, 16, 32 mg/kg, p.o.) selectively enhanced rSOWM performance only when the task difficulty level was high (3-stimulus, with 6-s delays). The specificity of the effects parallels the studies conducted in healthy volunteers using the human CANTAB SWM, where the cognitive enhancing effects of modafinil (200 mg) only occurred at the highest difficulty levels (e.g., 10-box level)(158), and highlights the potential of rSOWM as a translational tool to study WM. Moreover, we showed that the modafinil treatment results were robust and reproducible: we replicated our finding with similar effect size using two separate cohorts of rats that had different training histories and significant differences in baseline performance at time of modafinil administration. Interestingly, a lower dose of modafinil (16mg/kg) exerted the highest performance improvement in the cohort of rats with higher baseline performance; whereas, 32mg/kg was the dose presenting higher improvements in the cohort of rats with lower baseline performance. This suggests that modafinil dose effects may vary as a function prior training history.

Our studies using bilateral microinfusion of modafinil (0.2, 2.0  $\mu$ g/side) alone or after alpha-flupenthixol in the PFC suggested that the cognitive enhancing effect of modafinil may be mediated by D1 and/or D2 receptors located in mPFC. This was consistent with the known evidence that modafinil binds and inhibits the DA transporter and thus increases DA levels (173). While many human studies have shown that low doses of modafinil enhance multiple cognitive parameters, including WM, the mechanism of

action remains unclear. Modafinil's procognitive effects have been linked to a large number of neurotransmitter systems (174), greatly challenging the search for mechanism. Studies have shown that modafinil exhibits robust effects on catecholamines, serotonin, glutamate, gamma amino-butyric acid, orexin, and histamine systems in the brain (171). Most mechanistic studies to date have examined modafinil's effects in WM after systemic administration. Only one previous study (175) had intracranially administered modafinil in the hypothalamus to study wakefulness. Our study is the first to demonstrate that modafinil's nootropic effects may be through direct action in the mPFC, and depends upon intact dopaminergic receptor function. Consistent with the results after systemic administration, cognitive enhancing effects of bilateral mPFC infusions of modafinil were only observed in the harder task conditions (3 stimulus trials). This further highlights the importance and benefits of the capability to increase WM load present in the novel rSOWM task.

We also investigated the potential of mGluR5 Positive Allosteric Modulator (PAM) as a WM enhancer. mGluR5 receptors (mGluR5) are expressed in key brain regions for cognition, including hippocampus, amygdala, striatum and prefrontal cortex (176). Studies with the mGluR5 PAM ADX47273 reported both antipsychotic and precognitive effects (177). Gastambide et al., 2012 (178) confirmed that positive modulation of mGluR5 with LSN2463359 led to improvements in attention and flexibility in cognitively impaired animals within a rodent set-shifting paradigm. Recent studies indicate that selective activation of specific mGluR subtypes may provide potential benefits for the cognitive impairments observed in individuals with SCZ and other disorders (179, 180). Although multiple studies have been reported cognitive enhancing effects of mGluR5 PAMs in different domains of cognitive function (177, 179), the precise effects that the drug exerts over the specific WM domain remain unclear. The present study is among the first to assess the effects of LSN2463359 on WM performance. Our general lack of effect – with even potential performance impairment - observed with the mGluR5 PAM, suggests that rSOWM performance may not be sensitive to glutamatergic signaling via these receptors and suggests that the cognitive enhancement by modafinil is likely due to distinct mechanisms.

These pharmacological findings further suggest that the rodent SOWM test is a powerful and translational tool for elucidating the mechanisms of WM and executive function and for improving our preclinical drug discovery efforts.

### 6.3. mPFC 5-HT and DA modulation during rSOWM.

There is considerable evidence suggesting that DA is important for WM. DA release in the mPFC has been primarily studied with indirect and relatively slow techniques (e.g., voltammetry, microdialysis) in animals performing WM tasks. The role of 5-HT in WM is much less clear. In order to investigate the functional role of the monoamines, DA and 5-HT, within the PFC circuitry in WM, we used genetically encoded GRAB sensors to visualize 5-HT and DA dynamics *in vivo* (161, 181) during the rSOWM task. To my knowledge, these are among the, if not the first recordings, examining the phasic release of DA and 5-HT signals in the mPFC while mice are actively engaged in a visual working memory task.

*In vivo* fiber photometry recordings of mice injected with either GRAB DA3h or GRAB 5-HT2h in the mPFC indicated that DA and 5-HT release levels in mPFC were inversely modulated during the different trial events of the rSOWM paradigm. First, DA signal decreased when mice made stimulus touches. This decrease in DA signal was much more pronounced when the touch was correct compared to incorrect touches. Moreover, DA signal seconds before correct touches was increased compared to incorrect touches and baseline levels. In contrast, 5-HT release was increased at stimulus touch onset but did not show any differential modulation before correct or incorrect touches in rSOWM. Together, these data show that DA levels, but not 5-HT levels, in the mPFC may be predictive of subsequent correct touches.

We also found that DA release in the mPFC was transiently elevated during the early-delay period independent of trial type and performance outcome. A similar observation has recently been observed in a study not yet peer-reviewed (Biorxiv), where authors showed that DA signals in the mPFC were only transiently elevated during the early-delay period in performing an olfactory-based WM task (182). Interestingly, we also found that, after this transient elevation in DA release, DA signal during the delay period remained elevated before correct touches compared to incorrect touches; and this was again only observed in the more difficult task conditions (e.g., 3 stimuli, 6-s delay). These data are consistent with a large number of studies showing that WM function is impaired when dopamine (DA) D1 and/or D2 receptor occupancy within the PFC falls outside a critical range of an inverted U-shaped function (183, 184). These results are also consistent with our previous findings with modafinil and  $\alpha$ -flupenthixol suggesting that D1/D2 receptors are important for WM performance specifically in the most difficult rSOWM parameters. Furthermore, entropy measures of choice data in rSOWM indicated that animals expressed sequencing preferences only in the easier 2 stimuli trials. The fact that DA release signal is

increased before correct touches in 3 stimuli but not 2 stimuli could suggest that the increase in DA is necessary for the correct response only when WM is challenged and performance is not defaulted to preferred sequences. To our knowledge, this is the first data that, using a direct measurement approach, show that phasic DA release dynamics in mPFC during the delay are predictive of subsequent choice outcome in a WM task.

While the prefrontal DA signal during the delay was associated with WM performance outcome, 5-HT levels were observed to monotonically rise during the delay but were not differentially associated with choice success. This slow rise in 5-HT appeared independent of choice outcome. One of the main theories about how 5-HT regulates behavior is through its involvement in “waiting” or showing patience across delays. It has been previously shown that activation of dorsal raphe serotonergic neurons promotes waiting behavior or response inhibition (150, 151). One possibility is that the observed slow increase in 5-HT release during the delay period could perhaps be a mechanism by which 5-HT “tracks waiting time” before the next touch can be made and, therefore, the next reward can be obtained. Further analysis and manipulations need to be done to explore this hypothesis.

Intriguingly, an opposing dynamical pattern to reward collection was observed for prefrontal DA and 5-HT. There was a biphasic increase in gDA3h fluorescence and a biphasic decrease in g5HT2h in response to the reward cue and reward collection. Opposing roles of DA and 5-HT have long been suggested specifically in the context of motivation and reinforcement learning (130, 133) yet no direct measurements during WM have been reported to date. Contrary to the hypothesis of Daw, Dayan and colleagues (130), the opponency in phasic DA and 5-HT signals did not appear to relate to specific reward and punishment (or non-reward) conditions. Rather, the DA and 5-HT signals seem to be continuously bidirectionally opposed across behaviorally relevant events. A similar opposing modulation of prefrontal DA and 5-HT in response to reward has recently been shown during a looming task of anxiety but has not yet been peer reviewed (185). Future work using optogenetic or other stimulation techniques during GRAB sensor photometry recordings could help to better understand the casual interactions between phasic release of these two neurotransmitters.

## 6.4. The rodent Delay and Reward Discounting task

To better understand whether observed DA and 5-HT release changes during rSOWM are specific to the WM component (delay) and not uniquely related to reward prediction, we developed the rDRD task. In a rSOWM correct trial, the three main event phases in order are as follows: delay - touch - reward. The main goal with this project was to monitor prefrontal release dynamics of DA and 5-HT specifically during the WM delay. However, it is likely that a combination of simultaneous processes is taking place during and around the delay phase (e.g., response to the reward predicting cue, reward expectation, reward reinforcing response). In order to better characterize the specific DA and 5-HT release dynamics during the WM delay, we wanted to identify the differential neurotransmitter modulation occurring during the other task event phases when WM was not involved. To do so, we developed the rDRD task. In rDRD, mice can choose between images that are associated with subsequent reward delivery after a delay period: touch – delay – reward. In contrast with rSOWM in which the choice occurs after the delay period, in rDRD, the delay period takes place after the choice and is the waiting period before the reward is delivered. By comparing DA and 5-HT modulation during the three event phases across the two cognitive tasks, we aimed to identify the specific neurotransmitter dynamics in mPFC that are linked to WM.

DD in rodents is commonly assessed by two-choice operant tasks in which animals select between a small, immediate reward, and a larger, delayed reward. In most protocols, the magnitude and location of the small, immediate reward option typically remains fixed throughout a given session or experiment (186). This configuration makes it difficult to separate either sensitivity to differences in reward amount or side bias from actual choice preference based on value. On each DRD trial, two visual stimuli are presented side-by-side on a touch screen. Animals are then freely allowed to touch one and, after a programmed delay period, a liquid reward is delivered. Reward magnitude and delay on a given trial are uniquely dependent on the chosen stimulus. Each combination of reward size (15, 25, 35, 50 $\mu$ L) and delay (0, 2, 4 or 6s) are represented by the visual properties of 16 different images. Any systematic biases can be corrected by titrating forced choice trials.

The rDRD presents many advantages compared to classic DD tasks. First, many DD tasks use two fixed response locations (e.g., levers), one being associated with the smaller and the other with the larger reward. In rDRD, screen location of visual stimuli can be counterbalanced so that the smaller or larger reward is equally represented on both sides of the screen. This is important because it forces subject

animals to reevaluate the choice presented on each trial so that it is unlikely that they default to their “preferred side”. Second, in DD tasks the Smaller-Shorter (SS) option is typically fixed (e.g., starts at 0s). In rDRD, parametric variations of both delay and magnitude are performed. Third, multilevel logistic regression modeling can be applied to the choice data to estimate the separate parametric contributions of sensitivity to reward magnitude ratio and relative delay. Multilevel logistic regression modeling applied to our rDRD choice data revealed that mice show, in average, higher discounting based on delay length than reward magnitude. However, there was high heterogeneity and individual differences in reward and delay discounting rates. Lastly, rDRD is performed in a touchscreen chamber and thus, performance and experimental observations can be easily compared to other tasks using the same conditions.

*In vivo* fiber photometry recordings indicated that DA and 5-HT were oppositely modulated in the mPFC in response to stimulus touches and reward collection. This was a consistent finding in both rDRD and rSOWM tasks, which contributed to the strength and validity of the observation. Prefrontal DA and 5-HT release signals were sensitive to, and dependent on, delay and reward magnitudes. DA release dynamics were not different depending on the stimulus choice (Smaller-Sooner versus Larger-Later). On the contrary, higher levels of 5-HT preceding stimulus touch were associated with the choice of immediate rewards (no delay).

Prefrontal DA release monotonically increased at reward collection onset and the magnitude of the signal was dependent on the delay. The increase in duration in gDA3h fluorescence was also dependent on the reward magnitude chosen. The longer the magnitude, the longer the increased DA signals lasted. Consistent with our rSOWM data, the opposite pattern was observed for 5-HT. 5-HT signal was significantly reduced at reward collection. 5HT signal remained negatively modulated and below baseline for multiple seconds after reward collection and during the ITI period. The time window during which 5-HT2h signal remained below baseline was dependent on the reward magnitude collected. Together, these two findings indicate that prefrontal 5-HT signaling may be sensitive to the length of waiting time prior to the reward delivery, as well as the consumed reward size (151, 187). Although the involvement of 5-HT in reward processing is undisputed, this is the first time we can parametrically manipulate delay and reward to better understand its precise contribution by using one behavioral paradigm.

Lastly, data from gDA3h fiber photometry recordings in DLS served as an experimental control for our data using GRAB DA (gDA3h). Fiber photometry recordings in DLS showed that DA release strongly

increased in response to both the reward predicting cue and reward processing (163) as expected by the reward prediction error theory. Due to the increased concentration in DA in DLS, it was not surprising that DA signal changes in DLS were significantly larger (almost 10-fold), especially when compared to mPFC where dopaminergic projections are much more diffused.

Overall, I have developed and validated the behavioral parameters of two novel rodent cognitive paradigms that closely mirror paradigms used in humans and that can be used to understand the neural circuitries involved in WM and delay discounting related processes, such as impulsivity or risk aversion. Monitoring in vivo dynamic changes of prefrontal DA and 5-HT across both tasks developed in this thesis may serve as a powerful strategy to identify their specific contribution on WM, and time and reward processing.

## 7. Conclusions

1. Over the course of this thesis, I developed and validated two novel psychometric touchscreen tasks to assess working memory (rSOWM) and delay discounting processes (rDRD) in rodents. These tasks were designed as close analogues to human paradigms to enhance their translational value.
2. rSOWM performance was differentially disrupted by lesion of subregions of the hippocampus and was improved in a difficulty-dependent manner by systemic administration of the putative cognitive enhancer modafinil, mirroring results observed in humans. These results suggest that rSOWM is a robust rodent analogue of SWM providing a powerful translational tool for elucidating the mechanisms of WM and for improving preclinical drug discovery efforts.
3. This study is the first to demonstrate that local microinfusion of modafinil into mPFC can exert cognitive enhancing effects in a WM task. Moreover, these enhancements were selectively reversed by local infusion of a D1+D2 receptor antagonist suggesting that prefrontal DA plays a critical role in the nootropic effects of modafinil.
4. With the use of recently developed GRAB sensors in combination with fiber photometry techniques, we found opposing dynamics of prefrontal DA and 5-HT following key behavioral events (e.g., after stimulus choice and reward collection) in both cognitive paradigms. This is the first study that shows *in vivo* opposing phasic dynamics for these prefrontal monoamines.
5. These are the first data, to our knowledge, that demonstrates that phasic DA release dynamics in mPFC during the delay are predictive of subsequent choice outcome in a WM task. This result was selective to the more difficult trial types (e.g., 3 stimuli, 6-s delay) and was consistent with our modafinil study showing WM enhancements via DA receptors only when WM load is challenged.
6. Prefrontal 5-HT levels monotonically increased during the rSOWM delay but – unlike DA - were not differentially associated with choice outcome. Higher levels of 5-HT in the mPFC were associated with increased choice of immediate (no delay) over delayed rewards in rDRD.

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