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"Neurophysiological alternations in the Hippocampus after Working Memory Training"

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1. Abstract

Working Memory (WM) is the ability to store and adaptively handle information in a timescale of seconds, in order to plan and execute complex cognitive tasks Besides the prefrontal cortex (PFC) which has a pronounced role in this process, hippocampus also has a pivotal role -especially in spatial WM. Although the neural correlates of WM have been extensively researched, the neurobiological effects of WM training remain largely unknown. Previous work from the lab has shown that WM training in male mice improves cognitive flexibility and enhances long-term potentiation (LTP) as well as dendritic spine density in the PFC. On the other hand, WM training in female mice did not affect cognitive flexibility or the LTP and dendritic spine density in the PFC. However, it enhanced both LTP and dendritic spine density in the hippocampus. Therefore, this study aimed to extend the above results by investigating the effects of WM training in female mice in anxiety, WM reference memory as well as the properties of spontaneous activity immediately following the training. Furthermore, the study investigated the duration of the LTP enhancement in the hippocampus.

Adult female mice were separated in two training groups: one that was trained in the delayed alternation task (adaptive) and the other that was trained in the alternation procedure (non-adaptive) for 6 days. The effects of training on anxiety levels, using the open-field and light dark test, on working memory and on reference memory and reversal learning, using the left-right discrimination task and reversal were measured. Both groups showed reduced anxiety levels in the light-dark test following training, compared to before training. In the working memory test, the adaptive groups performed better compared to the non-adaptive group. In the left-right discrimination task, there was no difference in the performance following training, compared to before, but mice performed the task with reduced latency. In the reversal learning, both behavioral groups performed with higher accuracy and latency post-training. Following the behavioral testing, the mice brains were harvested for electrophysiological recordings, either immediately to study the properties of the spontaneous activity or 2 weeks later to investigate LTP. To study the properties of spontaneous activity, field potential recordings were taken from the three different subregions of the hippocampus, namely CA1, CA3 and dentate gyrus (DG). Our results showed elevated spontaneous spiking activity and oscillatory power in the hippocampus. Finally, the effects of training on synaptic

plasticity were observed 14 days after training, when field excitatory post-synaptic potential recordings were obtained in the CA1 region of the hippocampus. Both groups had enhanced long-term potentiation compared to naive controls. Taken together, these results suggest that working memory training leads to cognitive flexibility and enhances oscillation power as well as long-lasting synaptic plasticity in HPC.

keywords: working memory; working memory training; cognitive flexibility; hippocampus; spontaneous activity; long-term potentiation; plasticity

1.1. Περίληψη

Η μνήμη εργασίας είναι η ικανότητα να καταχωρούμε και να διαχειριζόμαστε πληροφορίες εντός δευτερολέπτων, με σκοπό τον σχεδιασμό και την εκτέλεση πολύπλοκων γνωστικών έργων. Εκτός από τον εξέχων ρόλο του προμετωπιαίου φλοιού (Prefrontal Cortex, PFC) σε αυτή την διεργασία, κομβικό ρόλο κατέχει και ο ιππόκαμπος (Hippocampus, HPC) - ειδικά στην χωρική μνήμη εργασίας. Ενώ το νευρωνικό υπόβαθρο της μνήμης εργασίας έχει μελετηθεί σε μεγάλο βαθμό, η νευροβιολογική βάση της επίδρασης που έχει η εκπαίδευση της μνήμης εργασίας παραμένει σε μεγάλο βαθμό άγνωστη. Προηγούμενες μελέτες από το εργαστήριο έχει δείξει ότι η εξάσκηση της μνήμης εργασίας σε αρσενικούς μύες βελτιώνει την γνωστική ευελιξία, ενισχύει την μακρόχρονη ενδυνάμωση (Long-Term Potentiation, LTP) και αυξάνει την πυκνότητα των δενδριτικών ακανθών στον PFC. Απεναντίας, αυτές οι αλλαγές δεν παρατηρήθηκαν στον PFC όταν πραγματοποιήθηκε η εκπαίδευση της μνήμης εργασίας σε θηλυκούς μύες, ενώ σημειώθηκε ενίσχυση LTP και αυξημένης δενδριτικής πυκνότητας στον HPC. Η παρούσα μελέτη στοχεύει να επεκτείνει τα παραπάνω αποτελέσματα, ερευνώντας σε θηλυκούς μύες την επίδραση της εκπαίδευσης της μνήμης στα επίπεδα άγχους, στην ανάστροφη μάθηση και στις ιδιότητες της αυθόρμητης δραστηριότητας αμέσως μετά την περίοδο εκπαίδευσης. Επιπλέον, ερευνάται και η διάρκεια που η ενίσχυση στο LTP του ιπποκάμπου μπορεί να διατηρηθεί.

Ενήλικοι θηλυκοί μύες χωρίστηκαν σε δύο ομάδες: μια που εκπαιδεύτηκε σε δοκιμασία εναλλαγής με καθυστερήσεις (delayed alternation task, adaptive group) και μια που εκπαιδεύτηκε στην διαδικασία εναλλαγής (alternation task, non-adaptive group) για 6-9 ημέρες. Η επίδραση της εκπαιδευτικής αυτής διαδικασίας στα επίπεδα άγχους ερευνήθηκε με τις δοκιμασίες ανοικτού πεδίου (Open Field test) και φωτεινού-σκοτεινού πεδίου (Light-dark test), στην χωρική μνήμη αναφοράς με την δοκιμασία διάκρισης μεταξύ βραχιόνων λαβυρίνθου με σχήμα T (left-right discrimination task) και στην γνωστική ευελιξία με την ανάστροφη μάθηση (reversal learning). Κατά τον έλεγχο της μνήμης εργασίας, μύες της ομάδας adaptive είχαν υψηλότερα ποσοστά επιτυχίας σε σχέση με μύες της μη-προσαρμοστικής. Στην δοκιμασία διάκρισης μεταξύ των βραχιόνων δεν παρατηρήθηκε διαφορά στην επίδοση των μυών μετά την εκπαίδευση, αλλά με μικρότερη περίοδο αδράνειας πριν την επιλογή (reduced latency). Σχετικά με την ανάστροφη μάθηση, παρατηρήθηκε ότι μετά την εκπαίδευση της μνήμης εργασίας και οι δύο ομάδες είχαν υψηλότερα ποσοστά επιτυχίας και μικρότερες

περιόδους αδράνειας πριν την επιλογή. Έπειτα από τις συμπεριφορικές δοκιμές, οι εγκέφαλοι των μυών απομονώθηκαν για ηλεκτροφυσιολογικές καταγραφές: είτε απευθείας για την μελέτη της αυθόρμητης δραστηριότητας, είτε 2 εβδομάδες αργότερα για την διερεύνηση LTP. Για την μελέτη της αυθόρμητης δραστηριότητας πραγματοποιήθηκαν καταγραφές πεδίου από τρεις υποπεριοχές του HPC: τις CA1, CA3 και την οδοντωτή έλικα (Dentate Gyrus, DG). Τα αποτελέσματα έδειξαν αυξημένα επίπεδα αυθόρμητης δραστηριότητας και νευρωνικών ταλαντώσεων στον HPC. Τέλος, η επίδραση της εκπαίδευσης της μνήμης εργασίας στην συναπτική πλαστικότητα παρατηρήθηκε 14 ημέρες μετά το τέλος της εκπαίδευσης. Και στις δύο ομάδες σημειώθηκε ενίσχυση του LTP σε σύγκριση με τα ζώα που δεν είχαν εκπαιδευτεί (naive controls). Συνολικά, τα παρόντα αποτελέσματα υποδεικνύουν ότι η εκπαίδευση της μνήμης εργασίας οδηγεί σε αυξημένη γνωστική ευελιξία, μεγαλύτερη ισχύ των νευρωνικών ταλαντώσεων καθώς και συναπτική πλαστικότητα που διατηρούνται μακρόχρονα.

Λέξεις κλειδιά: μνήμη εργασίας, εκπαίδευση μνήμης εργασίας, γνωστική ευελιξία, ιππόκαμπος, αυθόρμητη δραστηριότητα, μακρόχρονη ενδυνάμωση, πλαστικότητα

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3. Introduction

3.1 Working Memory: delineation from theoretical models

The ability to store and adaptively handle information is the functional backbone of cognition. This function is attributed to Working Memory. Definitions of this term have been under great revisions, as numerous theoretical models have contributed to conceptualizing it. It was firstly used by Miller et. al in 1960 to describe a capacity constraint of memorized content, useful to carry out behavior (G. A. Miller, Galanter, & Pribram, 1960). In 1968 it was described as part of a multi-unit memory system that included a sensory register, short- and long-term memory, as sensory inputs were transiently kept on-line to be processed by short-term memory (Atkinson & Shiffrin, 1968). Next, instead of conceiving working memory as a unitary scheme, subdivisions were introduced by Baddeley and Hitch in 1974. This model consisted of a central executive that controlled the integration of temporarily stored visuospatial and auditory information (Baddeley & Hitch, 1974). Later, Cowan pointed out the role of attention and the dynamic exchange from long-term to working memory, as well as its limited capacity (Cowan, 1998). Until today, working memory is subjected to constant redefinitions; however, the foundational role of working memory can be described as the cognitive function that utilizes maintenance and manipulation of short-term information, in a timescale of seconds, in order to plan and execute complex cognitive tasks, such as goal-directed behavior (Christophel et al., 2017; Stavroulaki, Giakoumaki, et al., 2021).

3.2 Neuroanatomical Circuitry Supporting Working Memory

Working Memory functions are supported by interactions among a distributed network of brain regions. The fundamental role of PFC in higher-order cognitive functions, primarily WM, has been undisputed. PFC is responsible for executive functions, such as decision-making, goal-oriented behaviors, cognitive control, and integration processes (Liu, Bai, Xia, & Tian, 2018b; Zhang, Guo, & Liu, 2022b). Early lesion studies and subsequent impaired WM performance have supported that dorsolateral PFC (dlPFC) is implicated in spatial WM (Levy & Goldman-Rakic, 1999). Later technological advances in neurophysiological recordings and expanding research on non-human primates proved that PFC functions are not limited in spatial WM. Prefrontal neurons are also selectively activated regarding the stimulus properties and differentiate their firing rates upon levels of abstraction (Riley & Constantinidis, 2015).

As Mark D'Esposito has stated, it appears that PFC functional interactions with the rest of the brain is the source of WM emergence (D'Esposito, 2007).

Additionally, it has been proven that hippocampus (HPC) has critical involvement in WM, apart from long-term memory (LTM) and the spatio-temporal context in which they occur (Jaffe & Constantinidis, 2021). Hippocampal neurons exhibit activation during WM tasks, as well as stimulus selectivity for spatial WM tasks (Jaffe & Constantinidis, 2021). This is founded upon anatomical and functional interactions between PFC and HPC, which consist of direct monosynaptic and indirect connections (Zhang, Guo, & Liu, 2022a). Disruptions in either the PFC or the HPC have proved to negatively affect performance in WM tasks (de Mooij-van Malsen et al., 2023; Yangguang Ou, Rachael E Wilson, 2018). On the other hand, higher synchronicity levels between PFC-HPC enhance WM performance (Liu et al., 2018a).

The initial notion that WM contents are stored and processed by the PFC, has been enriched by studies in humans and non-human primates that have observed activation in sensory cortices during WM tasks (Christophel et al., 2017; Stavroulaki, Giakoumaki, et al., 2021). This implies that properties of WM contents are systematically deconstructed and represented in areas specialized in the respective content. In particular, activations have been observed in the parietal and premotor cortex that contribute to sensory processing and motor planning respectively, in order to meet the requirements of WM demands (Stavroulaki, Giakoumaki, et al., 2021). For instance, visual or auditory WM contents lead to activations in the occipital and temporal lobes respectively, along with the premotor cortex and basal ganglia for movement preparation. Processing these deconstructed components is also found to be mediated by the midline thalamus, which is also closely connected with PFC and HPC (Bueno-Junior & Leite, 2018).

Overall, a Working Memory gradient appears to exist from higher to lower hierarchy areas with each one of them having a different functional role regarding WM processing: the features of incoming WM information are represented at different levels of abstraction throughout the brain, in order to eventually plan a suitable behavioral response, all across a temporal delay. The extent of the involvement of each region is dependent on the formats suitable to execute the WM requirements. This redundancy of different representations of WM contents within this network allows for precise and efficient computations, accounting for the robustness of this process (Christophel et al., 2017).

3.3 Neural Mechanisms

Persistent Activity

As a core component of cognition, extended research has focused on understanding the neural substrates of working memory. Early studies showed that the PFC was high in the hierarchy in the circuitry that supported working memory (Goldman-Rakic, 1996). Since then, further evidence supported its predominant role in WM as well as its supporting cellular mechanisms (Riley & Constantinidis, 2015). PFC neurons appear to maintain their stimulus-evoked activity in a period after the stimulus is no longer present, termed as "persistent activity" (Constantinidis et al., 2018; Jaffe & Constantinidis, 2021). In neurophysiological experiments in non-human primates as well as in humans, persistent activity is postulated to reflect the active representation of task-relevant information (not only the retrospective representation of the stimulus, but also a prospective action plan), since the behavioral outcome could be predicted based on these repeated discharges (Christophel et al., 2017; Constantinidis et al., 2018). Even though this mechanism is greatly investigated in the PFC, persistent activity is a wide-spread cortical phenomenon and provides parallel processing of incoming WM information in different levels of complexity and abstraction (Christophel et al., 2017). Apart from the PFC, there are indications of emergence of persistent activity also in the posterior parietal cortex, the entorhinal cortex, the thalamus, and the striatum as well as the hippocampus (Boran et al., 2019; E. K. Miller, Lundqvist, & Bastos, 2018; Stavroulaki, Giakoumaki, et al., 2021).

Rhythmicity model

However, another neural mechanism of WM has been put forward that relies on a different aspect of neural activity. The rhythmicity model proposes that WM processes are mirrored by the unified activation of neuron populations (Jaffe & Constantinidis, 2021). These streams of rhythmic coactivation are referred to as "neural oscillations". They are a core intrinsic property of neuronal ensembles and permit the flow of information from local to large-scale networks (Fries, 2015, Gansel, 2022, Buzsaki & Watson, 2012). Neural oscillations can be divided based on frequency as follows Delta: 1–4 Hz, Theta: 4–7 Hz, Alpha: 8–12 Hz, Beta: 13–30 Hz, Gamma: 30–80 Hz and High Gamma: 80–200 Hz. It has been observed that cognitive processes can modify the properties of network oscillations, such as phase (activation timing) and amplitude (squared amplitude is the oscillatory power, the number of synchronously active

neuronal units) that modulates the underlying neuronal processing and coding (Arski, Young, Smith, & Ibrahim, 2021). The magnitude, frequency, and phase of population activity in WM-implicated brain regions and their modifications over task-epochs, have been found to be modified depending on the underlying working memory process (Constantinidis et al., 2018; Jaffe & Constantinidis, 2021; Lundqvist et al., 2016). Synchronicity is also an indicator of functional connectivity among brain regions that contribute to support WM function (D'Esposito & Postle, 2015).

A different activity-silent mechanism of WM is based on synaptic mechanisms. Computational models have argued that WM information storage can depend on changes in synaptic weights. This is founded on the notion of repetition suppression: the reduction of stimulus-elicited activity when its presentation is repeated (Jaffe & Constantinidis, 2021). Such activity patterns may not result in spike generation, may not have an impact on spiking activity, but on synaptic strength. This can be performed by short term facilitation of vesicle release or short-term potentiation of synaptic terminals. (Constantinidis et al., 2018; Jaffe & Constantinidis, 2021; Mongillo, Barak, & Tsodyks, 2008).

It is plausible these diverse mechanisms are interlaced to carry out different attributes of WM tasks (Stavroulaki, Giakoumaki, et al., 2021).

Consistent with the principles of perception, there is a limit of the input that the brain can process simultaneously. Similarly, WM can hold online a certain amount of information, a feature referred to as "capacity" (Jaffe & Constantinidis, 2021). This is not a fixed attribute, as has been proven that can decline with age or conversely broadened with training (Chung et al., 2021; Constantinidis & Klingberg, 2016; Stavroulaki, Giakoumaki, et al., 2021)

3.4 Spontaneous activity & Working Memory

Spontaneous brain activity refers to intrinsic neuronal activations in the absence of external stimuli or explicit tasks and constitute the basis of functional brain organization mirroring its dynamic nature (Tozzi, Zare, & Benasich, 2016, Kirkby et al., 2013). During circuit formation, patterned spontaneous activity ensures the establishment of precise wiring among neurons (Kirkby et al., 2013). It has been speculated that this process is based upon Hebbian-based learning principles, which claim that formation

of neural connections depends on repeated sustained activation of the implicated synapses; conversely, sparsely interacting synapses will gradually decay (Hebb, 1949, Katz and Shatz, 1996, Kirkby et al., 2013). However, additional non-Hebbian mechanisms might also contribute (Kirkby et al., 2013). Therefore, modifications of synaptic strength among neurons dictate the spatiotemporal dynamics of circuit spontaneous activity (Lonardoni et al., 2017).

Measuring spontaneous activity can be a significant indicator of specific functions and varied topology. It is the foundation of resting-state fMRI, - a widely used technique offering valuable insights on human brain functional connectivity (Raimondo et al., 2021). It has been recently proposed that spontaneous activity can provide insights on cognitive processes (Liu et al., 2022).

As mentioned above, WM is subserved by interactions of large-scale brain networks. This interregional communication is maintained by neural oscillations (Buzsáki & Watson, 2012). Numerous studies have proven that PFC-HPC oscillation synchrony is essential for WM function (Liu et al., 2018a; Yangguang Ou, Rachael E Wilson, 2018; Zhang et al., 2022b, 2022a). PFC is connected with the ventral-HPC (vHPC) by direct monosynaptic input and mPFC is indirectly connected with the dorsal-HPC (dHPC) via the nucleus reuniens of the thalamus (Bueno-Junior & Leite, 2018; de Mooij-van Malsen et al., 2023; Spellman et al., 2015; Zhang et al., 2022a). More specifically, theta oscillations are primarily observed in the hippocampus, which function as a pace-maker of PFC activation during WM. Moreover, long-range theta activity between frontal and temporo-parietal regions has been observed to account for attention and prioritization of relevant information in WM processing (Arski et al., 2021). Alpha oscillations dominate sensory cortices and thalamic activity. Recordings have indicated that theta-alpha coupling is evident during maintenance of WM. Concerning Gamma oscillations, their synchrony with theta activity underlie sequential WM information, whereas gamma-alpha coupling occurs when maintenance of multiple, sensory-spatial WM items is needed (Arski et al., 2021; Roux & Uhlhaas, 2014). Gamma frequency also increases with additive memory load (the number of items to be retained in WM) (Gu, van Rijn, & Meck, 2015). Other studies have proposed that peak theta and alpha frequencies are positively correlated with WM capacity (Gu et al., 2015). Additionally, beta oscillatory activity was firstly associated with motor processing, but studies have pinpointed its role in attendance of relevant information during WM (de Mooij-van Malsen et al., 2023). Delta, Theta, Beta are low-frequency oscillations and are

considered to convey temporal dynamics in WM retention (Gu et al., 2015). It is evident that spontaneously occurring brain activity during WM performance relies on multi-regional complex interactions that orchestrate the maintenance and manipulation of WM information.

3.5 Effects of Working Memory Training

The progressive improvement of performance in WM tasks has spurred a great amount of research, investigating whether other cognitive abilities can be enhanced by WM training as well (Stavroulaki, Giakoumaki, et al., 2021; von Bastian & Oberauer, 2014). Testing span types of WM (limits of WM duration and capacity) and adaptability (adjustment of task difficulty based on the trainee's performance, such as the n- back task) are the main branches of WM training. Comparison of behavioral measures before and after WM training have demonstrated post-training enhancements on cognitive skills in non-trained WM aspects, a phenomenon termed as "transfer" (Stavroulaki, Giakoumaki, et al., 2021). Notably, studies have shown improvements in fluid intelligence and executive functions (such as reasoning and inhibition control) in humans. The advances of WM training are considered to be beneficial, not only for cultivating the cognitive properties of healthy individuals, but also for age- and pathology-related cognitive deficits (psychiatric disorders, such as schizophrenia, major depressive disorder, cognitive impairments, such as Attention-Deficit/Hyperactivity Disorder (ADHD), traumatic brain injury and strokes, neurodevelopmental disorders such as Multiple Sclerosis, neurodegenerative diseases e.g. Alzheimer's and Parkinson's Disease) (Chai, Abd Hamid, & Abdullah, 2018; Stavroulaki, Giakoumaki, et al., 2021; von Bastian & Oberauer, 2014).

Given these indicated effects of WM training, it is of great importance to gain insight into the neural alterations that take place in the process. Human studies have shown brain-wide reorganization of neuronal activity (frontal and parietal cortices, striatum) alongside with white matter density in the anterior body of corpus calosum, a structure that connects the dorsolateral PFC between hemispheres Stavroulaki, Giakoumaki, et al., 2021). Non-human primate research has offered significant insights regarding delay activity: higher number of activated neurons, decreases in firing rate variability, decreases in noise correlation, implying changes in plasticity and information processing (Jaffe & Constantinidis, 2021).

3.6 Effects of Working Memory Training in Rodents

Rodents, as one of the most frequently used animal models in research, have provided valuable insights regarding cognitive processes. Rodent medial PFC (mPFC) comprising prelimbic and infralimbic mPFC, exhibits homology to the mPFC and Anterior Cingulate Cortex (ACC) in primates (Jung & Carlén, 2021, Laubach, Amarante, Swanson, & White, 2018). Numerous studies in rodents have proven their ability to perform WM tasks and observed activations in the same brain areas as primates, revealing significant aspects of the neural dynamics supporting WM (de Mooij-van Malsen et al., 2023; Light et al., 2010; Rossi et al., 2012; Song et al., 2020; Stavroulaki, Giakoumaki, et al., 2021; Stavroulaki, Ioakeimidis, et al., 2021; Szechtman et al., 2017; Yangguang Ou, Rachael E Wilson, 2018; Zhang et al., 2022b). WM mechanisms have also been characterized with commonalities between rodents and primates: persistent/delay activity as well as hippocampal contribution to WM processes have been documented (Jaffe & Constantinidis, 2021). Modulation of oscillatory activity during WM performance has also been exhibited during execution of WM tasks, whereas disruptions of those lead to impaired WM performance (Etter, van der Veldt, Choi, & Williams, 2023; Tamura, Spellman, Rosen, Gogos, & Gordon, 2017). Changes in cognitive flexibility after WM training were observed in male but not female mice. Regarding training-induced plasticity, increased LTP in PFC was evident in male but not female mice when recorded immediately after training. As for the HPC, males showed higher evoked responses in CA1-to-CA3 synapses compared to controls while only females exhibited LTP. Morphological alternations were also evident: male mice had increased spine density in the PFC and mature spines in the HPC, while increased spine density in HPC was found in females. These observations propose distinct WM training effects based on sex (Stavroulaki et al., 2020, Stavroulaki et al., unpublished data). On a cellular level, WM training has been found to induce long-lasting molecular changes in mice, such as differential expression of genes responsible for epigenetic alterations, neurogenesis, neuroplasticity and regulation of neuromodulators (Dopamine) (Stavroulaki, Giakoumaki, et al., 2021).

4. Aim of study

Based on our previous work, the aim of this study is to 1) investigate the effects of Working Memory Training on anxiety, working memory, reference memory and reversal learning in female mice that have undergone training in the delayed alternation task for WM training and 2) investigate the effects of WM training on the properties of spontaneous activity in HPC of female mice, as well as the long-lasting changes it causes on HPC plasticity.

5. Materials and Methods

5.1 Animals

All mice were bred and housed in the Dept of Biology, University of Crete facility. Female C57/B6 mice, aged 5-7 months old were housed in groups (2–4 per cage) and provided with standard mouse chow and water ad libitum, under a 12 h light/dark cycle (light on at 8:00 a.m.) with controlled temperature (24±1^{o}C). All animal procedures comply with the ARRIVE guideline, were performed according to the European Union ethical standards and University of Crete ethical rules and were approved by the Department of Biology, University of Crete-Experimental Protocol Committee.

5.2. Behavioral tasks (general aspects)

All mice were handled by the experimenter for at least 10 days to reduce the effects of stress by his/her presence. Furthermore, the mice were placed in the experiment room 1h prior to the experiment stable temperature at 22°C and light conditions for acclimatization. Before and after the use of each behavioral apparatus by each mouse, it was thoroughly cleaned with 70%v/v ethanol to prevent bias based on olfactory cues. Each experimental procedure videotaped for subsequent analysis with the behavioral analysis software, JWatcher® version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sydney, Australia. Available at http://www.jwatcher.ucla.edu/).

5.3. Anxiety behavioral tasks

To measure the levels of anxiety before and after the training protocol, we used two behavioral paradigms: the Open Field Test (OFT) and the Light-Dark Test. Both of these tests rely on the inherent aversion of mice to bright illuminated and exposed areas, alongside with their natural tendency to explore new environments (Kraeuter, Guest, & Sarnyai, 2019; Takao & Miyakawa, 2006).

The OFT is used to analyze locomotion, anxiety, and stereotypical behaviors such as grooming and rearing which indicate the general well-being of the mouse. Mice exhibiting a preference to remain close to the walls and to walk along the periphery show increased thigmotaxis (tendency to be in close proximity with a solid object). This is more prominent in mice showing signs of anxiety-like behavior, whereas mice with lower anxiety tend to spend more time in the center of the open field. For the OFT, each mouse was placed in the behavioral apparatus, located in a room with dim lighting, and

left to explore the new environment for 15 minutes. The time spent in each zone of the open field (walls, periphery, edges, center) was quantified to determine the stress levels of each mouse.

Following a 1h interval, the mice were tested in the Light-Dark Task. The apparatus was a rectangle cardboard box with two chambers divided by a wall with an opening, placed in a brightly lit experimental room. At the beginning of the procedure the opening was blocked, and the mouse was placed in the dark chamber for 10 seconds. After that, the object blocking the opening was removed and the mouse was left free to explore both chambers for 5 minutes. The total time spent in each chamber as well as the latency of entering the light department were measured before and after the working memory training to further assess the anxiety levels of the mice.

5.4. Cognitive tasks performed in the T-maze

To train working memory, we used the delayed alternation task in the T-maze. The T-maze apparatus included a start arm and two goal arms (45X5cm each). Mice were habituated in the T-maze apparatus, for 2 days. Mice were food-restricted so that the animals maintained 85-90% of their initial weight.

5.4.1. Left-Right Discrimination & Reversal Learning

One day after the habituation with the experiment apparatus, the preference of each mouse for one of the two arms was determined by measuring the number of times it chose to enter it and how many times it received the reward. After a 20-minute interval, the reward was placed in the opposite arm to test the reversal learning ability of each group. Following a 20-minute interval, one session of 10 trials was conducted, where the reward was placed in the opposite arm to test the reversal learning ability of each group.

5.4.2. Working Memory Training

All mice were subject to 10-trial sessions, 3 sessions/day. At the first trial of each session, mice were allowed to freely choose between the right or left goal arms. In the following trials, mice had to alternate the goal arms in order to receive reward, initially with no temporal delay between the trials. Once they reached a predefined criterion for the alternation procedure [i.e., 2 consecutive sessions of ≥70% correct choices

(performance)], mice were split into the non-adaptive and adaptive groups. Mice in the non-adaptive group continued to perform the same alternation task for 3 sessions per day. Mice in the adaptive group started the delayed alternation procedure, for which delays were introduced, initially at 10 seconds and increasing by 10 seconds when the criterion for each delay was achieved, for 6-9 days.

One day after the last training session, the mice performed 3 sessions of variable delays followed by a 10-minute interval. Each session consisted of 10 trials with a distinct delay duration. Mice which underwent 6 days of training were exposed to 20, 15, 10 second delays, in that order, while mice who had been trained 9 days performed under 30, 10, 20 second delays. In total, from the adaptive group 7 mice performed sessions with 10 seconds delay, 4 with 15 seconds, 7 with 20 seconds and 3 with 30 seconds. From the non-adaptive group 10 mice performed sessions with 10 seconds delay, 7 with 15 seconds, 10 with 20 seconds and 3 with 30 seconds.

5.5 Brain Slice Preparation

Mice were euthanized under halothane anesthesia. The brain was removed and placed in ice cold oxygenated artificial cerebrospinal fluid (aCSF) (95%O_2/5% CO_2) containing (in mM): 125 NaCl, 3.5 KCl, 26 $NaHCO_3$, 1 $MgCl_2$ and 10 glucose (pH =7.4, 315 mOsm/l). The brain was blocked and the part containing the hippocampus was glued onto the stage of a vibratome (Leica, VT1000S, Leica Biosystems GmbH, Wetzlar, Germany). The brain slices (400μm) containing the HPC were placed in a submerged chamber containing oxygenated (95% O_2/5% CO_2) aCSF (in mM): 125 NaCl, 3.5 KCl, 26 $NaHCO_3$, 2$CaCl_2$, 1 $MgCl_2$ and 10 glucose (pH = 7.4, 315 mOsm/l) at 36,6 ^oC. The brain slices were allowed to equilibrate for at least 1 h in this chamber before recordings began. The slices were placed in a temperature-controlled slice chamber at 36,6 ^oC with 95% O_2-5% CO_2 under a stereoscope continuously perfused initially with control aCSF containing (in mM): 125 NaCl, 3.5 KCl, 26 $NaHCO_3$, 2$CaCl_2$, 1 $MgCl_2$ and 10 glucose (pH = 7.4, 315 mOsm/l), followed by high K^+ aCSF (aCSF containing, in mM: 125 NaCl, 7.5 KCl, 26 $NaHCO_3$, 1 $MgCl_2$, 2 $CaCl_2$ and 10 glucose (pH = 7.4, 315 mOsm/l).

5.6. Spontaneous activity recordings

The recording electrode was filled with 3 M NaCl and was positioned in CA1, CA3 or DG regions of distinct hippocampal brain slices. Spontaneous local field potentials

(LFPs) were amplified using the EXT-02F amplifier (National Instruments), digitized with ITC-18 (Instrutech, Inc.) and recorded in a computer running Windows10 with WinWCP software (Stratchclyde electrophysiology software). In order to measure the number of spontaneous activity events the selectable high pass filter was adjusted at 3 Hz, to remove any offsets and to eliminate line frequency noise. When studying the oscillatory activity, the selectable high pass filter was adjusted at 0 Hz. The signal was low-pass filtered at 300Hz, in both types of recordings.

Regarding the measurement and analysis of the spontaneous activity events, 30 spontaneous voltage traces of 32 s duration were acquired under the experimental conditions described above. In off-line analysis a spontaneous event was identified as any voltage response larger than $5 \cdot \sigma b$. We calculated the frequency of spontaneous events by measuring the number of spontaneous events divided by the duration of the trace (32 s).

The detection of the oscillatory activity was achieved by acquiring the time series from the spontaneous voltage traces and transforming them into frequency domains using a fast Fourier Transform (FFT) algorithm. Using the FFT function, the Power Spectral Density (PSD) was estimated and the power spectrum of the oscillations, for each frequency domain, was computed in logarithmic scale. The percentage of the power spectrum from each frequency domain in relation to the power spectrum of the whole signal, defines a ratio referred as the rate of power (%). The rate of power (%) is estimated by the following equation:

$$Rate of Power(\%) = \sum \left(\frac{Power of X oscillatory domain}{Total Power}\right) * 100$$

The different frequency domains for which the ratio of the power spectrum was determined were Delta: 1–4 Hz, Theta: 4–7 Hz, Alpha: 8–12 Hz, Beta: 13–30 Hz, Gamma: 30–80 Hz and High Gamma: 80–200 Hz. All analysis was generated using custom-written procedures in MATLAB-R2015b (The MathWorks, Inc.).

5.7. Long Term Potentiation (LTP) recordings

In a different cohort of mice, 14-19 days following the end of the delayed alternation task protocol, mice were prepared for electrophysiological experiments using the *in vitro* slice preparation. We did not observe any differences dependent on the day of the recording. Mice were decapitated under halothane anesthesia. The brain was removed

immediately and placed in ice cold, oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 3.5 KCl, 26 $NaHCO_3$, 1 $MgCl_2$ and 10 glucose (pH=7.4, 315 mOsm/l). The brain was blocked and glued onto the stage of a vibratome (Leica, VT1000S, Leica Biosystems GmbH, Wetzlar, Germany). Brain slices (400μm thick) of the hippocampus were taken and were transferred to a submerged chamber, which was continuously super-fused with oxygenated (95% O2/5% CO2) aCSF containing (mM): 125 NaCl, 3.5 KCl, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$ and 10 glucose (pH=7.4, 315mOsm/l) in room temperature. The slices were allowed to equilibrate for at least an hour in this chamber before experiments began. Slices were then transferred to a submerged recording chamber (Scientifica, Inc), which continuously super-fused oxygenated (95% O2/5% CO2) aCSF containing (in mM): 125 NaCl, 3.5 KCl, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$ and 10 glucose (pH=7.4, 315mOsm/l) in room temperature.

The extracellular recording electrode, filled with NaCl (2M), was placed within the stratum radiatum of the CA1 hippocampal region. The platinum/iridium metal microelectrode (Harvard apparatus UK, Cambridge, UK) was also placed within the stratum radiatum layer of the CA1 region of the hippocampus, about 300μm away from the recording electrode, and was used in order to evoke fEPSPs. Responses were amplified using an EXT-02F amplifier (National Instruments), digitized using the ITC-18 board (Instrutech, Inc) on a PC using custom-made procedures in IgorPro (Wavemetrics, Inc, Lake Oswego, OR, USA). Data were acquired and analyzed using custom-written procedures in IgorPro software (Wavemetrics, Inc, Lake Oswego, OR, USA).

The electrical stimulus was generated equipped with a stimulus isolation unit (World Precision Instruments, Inc). The fEPSP amplitude was measured from the minimum value of the voltage response compared to the baseline value prior to stimulation. The fEPSP slope was measured from the point that the trace intersected 0mV until 1msec after. Both parameters were monitored in real-time in every experiment. A stimulus-response curve was then determined using stimulation intensities between 0.1-0.3 mA. Baseline stimulation parameters were selected to evoke a response of 1mV. LTP was induced using theta-burst stimulation, which consisted of 5 pulses at 100Hz, repeated four times at theta-rhythm (every 200ms). This stimulation was repeated three with an inter-stimulus interval of 20 seconds. Synaptic responses were normalized to the average 10 minutes pre-stimulus (theta-burst).

5.8. Statistical Analysis

All data groups were tested for normality. If they followed a normal distribution, t-test and one-way ANOVA including post hoc Tukey (HSD) test within groups were used. Statistical analysis was performed with GraphPad Prism v.10. Data are presented as mean ± standard error of mean (SEM) along with dot plots.

6. Results

6.1. Working Memory Training and Performance

For the behavioral experiments 18 adult female mice were used. Mice in the non-adaptive (n=10) group performed the alternating task without any delays, while mice in the adaptive (n=8) group underwent the delayed alternation task in the T-maze. Three of the adaptive and three of the non-adaptive group performed the delayed alternation experiment for 9 days.

The adaptive group needed an average of 10 sessions to reach criterion for the first time (n=8) and enter the delays phase. After that, an average of 6 and 5 sessions were needed to increase the delay duration to 20 and 30 seconds respectively. The number of mice reaching 20s delay were 6, though 3 of them proceeded to 30s delays. The non-adaptive group maintained above chance performance throughout the training period (Figure 1A,B).

In order to test the WM performance of both training group, 3 sessions of different delays in each section were used. The adaptive group performed significantly higher from the non-adaptive at the 10 second delay sessions (two tailed unpaired t-test, p=0,0044). In sessions with longer delays, no significant differences were measured between the two groups (two tailed unpaired t-test: 15 second delay: p=0,1722, 20 second delay: p=0,8302, 30 second delay: p=0.6702).

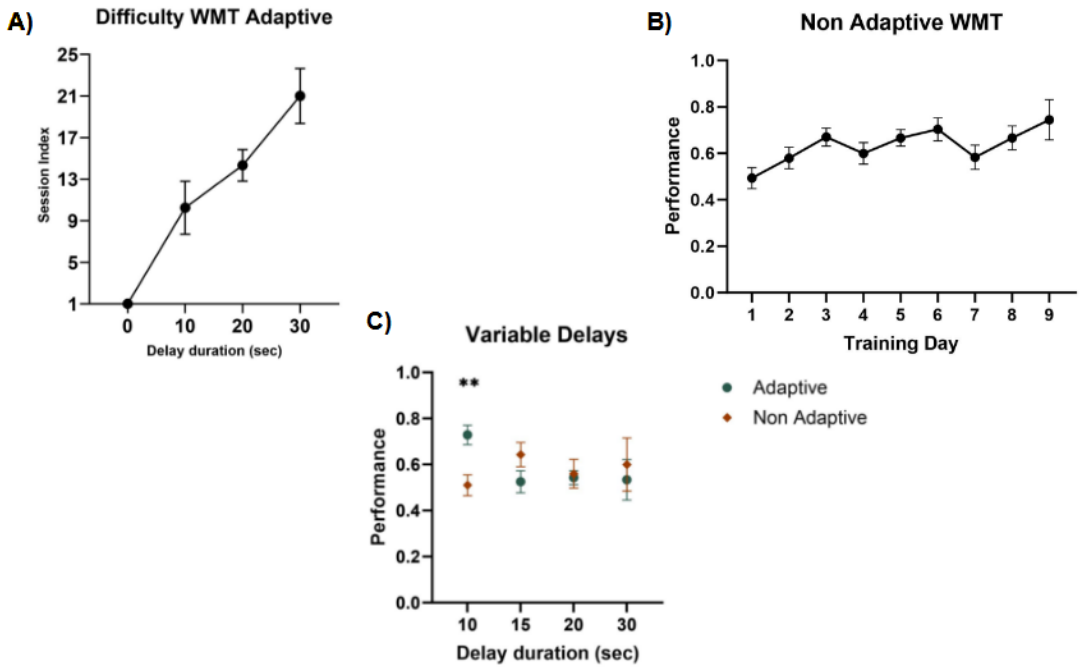


Figure 1. Working memory training performance

A. Graph indicating performance at different delay increments of the adaptive group during training, B. Average daily performance of the non-adaptive group, C. Graph showing performance in the delayed alternation task for both the adaptive and non-adaptive groups. The adaptive group outperformed the non-adaptive at the 10s delay in the variable delays test but did not exhibit significantly different performance on the rest of the delays (two tailed t-test: 15 second delay: p=0,1722, 20 second delay: p=0,8302, 30 second delay: p=0.6702).

6.2. Working Memory Training Effect on Anxiety Levels

Female mice were tested on the open-field and the light-dark test before and after WM training. In the Open Field Test, mice of the adaptive and the non-adaptive groups did not show any difference in the thigmotaxis index post-training, compared to pre-training (two-tailed paired t-test, adaptive: p=0,67, non-adaptive: p=0,18) (Figure 2A). In the Light Dark Test, there was no difference in the time spent in the light department after, compared to before, training for both groups (two-tailed t-test, adaptive: p=0,82, non-adaptive: p= 0,58). However, both the adaptive and non-adaptive groups exhibited significantly reduced latency to enter the light compartment after WM training, compared to before (two-tailed paired t-test, with p= 0,001 and 0,019 for the adaptive and the non-adaptive mice, respectively).

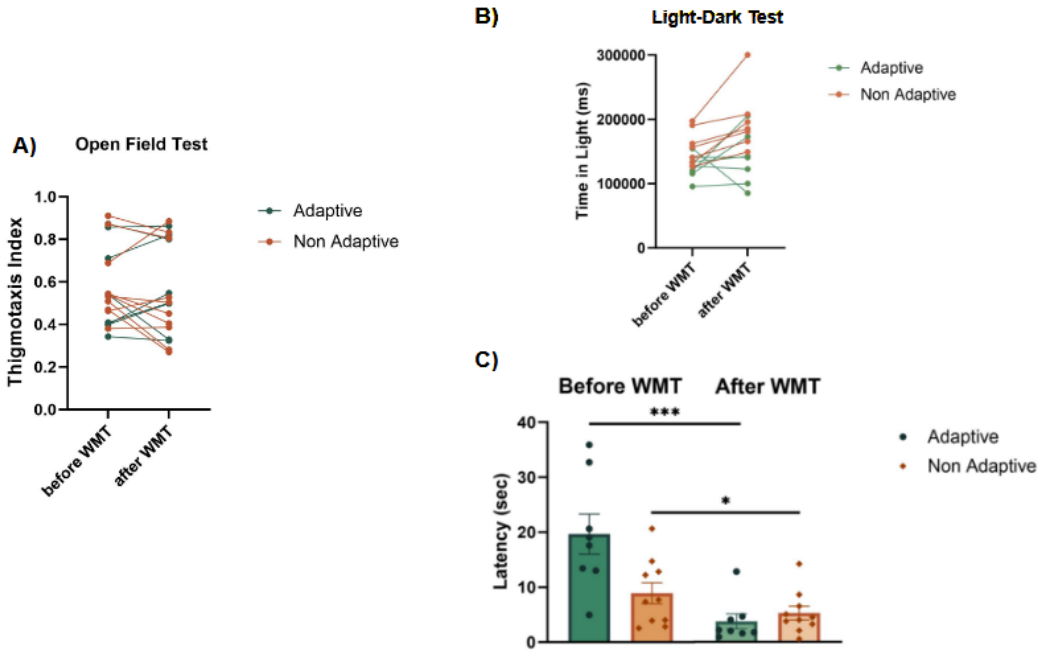


Figure 2. Stress response test before and after training

A. Graph showing the thigmotaxis index before and after WM training for the adaptive and the non-adaptive groups. There was no significant difference between the pre- and post-training thigmotaxis index for the adaptive (two-tailed t-test, p=0,67) and the non-adaptive (p=0,18) groups B. Graph showing the time spent in the light compartment before and after WM training for the adaptive (two-tailed t-test, p=0,82) and the non-adaptive (p=0,58) groups; no statistical significance was found between them (p=0,55). C. Graph showing the latency to enter the light compartment before and after WM training for the adaptive and the non-adaptive groups (two-tailed paired t-test, with p= 0,001 and 0,019 for the adaptive and the non-adaptive mice, respectively).

6.3. Left-Right Discrimination

In the Left-Right Discrimination Task, mice had to learn to go to a single arm in order to get a reward. There was no significant difference in the performance in the Left-Right Discrimination Task after WM training compared to before, for both the adaptive and the non-adaptive groups (two-tailed paired t-test, adaptive: p=0,08, non-adaptive: p=0,34). However, the latency to enter the chosen arm was significantly reduced after training, compared to before, in the non-adaptive group (two-tailed paired t-test with p=0,008). The adaptive group did not show a significant difference in the latency after, compared to before, WM training (two-tailed paired t-test, p=0,22).

6.4. Reversal Learning

In the reversal learning, both groups performed statistically significant higher numbers of correct arm choices after working memory training (paired t-test, p=0,03 and p=0,04, for the adaptive and non-adaptive group respectively) and significantly lower latency of making their choice (p=0,02 and p=0,009, for the adaptive and non-adaptive group respectively).

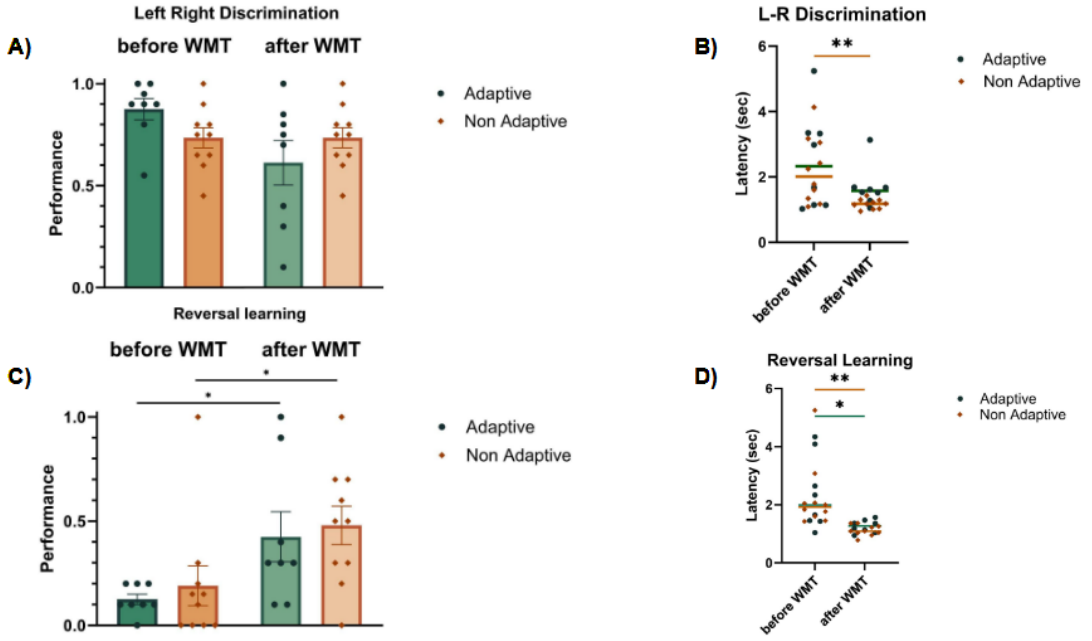


Figure 3. WMT effects on Left-Right discrimination and cognitive flexibility

A. Lift-Right Discrimination Test performance was not found to differ significantly before and after training for the adaptive and non-adaptive groups (two-tailed paired t-test, adaptive: p=0,08, non-adaptive: p=0,34); however, a robust increase of correct responses at the reversal learning task was evident in both groups (paired t-test, p=0,03 and p=0,04, for the adaptive and non-adaptive group respectively), B. Choice latency for the discrimination task did not appear to differ for the adaptive group ((two-tailed paired t-test, p=0,22), but was significantly lower for the non-adaptive group after training (two-tailed paired t-test with p=0,008), while both groups' latencies at the reversal learning task were significantly reduced (p=0,02 and p=0,009, for the adaptive and non-adaptive group respectively)

6.5 Effects of WMT on spontaneous activity of HPC

6.5.1. Spontaneous Spiking Events

Immediately following training, we investigated the frequency of spontaneous events in acute brain slices in the 3 HPC subregions, namely CA1, CA3 and DG. This time, we also included a naive group, that is mice that were not exposed to any kind of behavioral training. The frequency of spontaneous events was measured in two conditions: a) using control aCSF and b) using aCSF with increased K^+ concentration (High K+) which enhances spontaneous activity.

- **CA1**

The frequency of spontaneous spiking events was significantly different among the 3 groups in the CA1 region during control aCSF application (one-way ANOVA, F=5,131, p=0,0165). There was no significant difference in the number of spontaneous spiking events in the adaptive group, compared to the naïve group. (Tukey's Test, Adaptive vs Control, p-value= 0,54). On the other hand, the non-adaptive group showed statistically significant greater numbers of spontaneous events compared to the naive group (Tukey's test: Non-adaptive vs. Control, p=0,0125) (Figure 7A).

During High K^+ aCSF perfusion, no significant changes in the numbers of spiking events were identified among the three groups (one-way ANOVA, F=0,012, p=0,9874) (Figure 7A). Combining the data of the adaptive and non-adaptive groups and comparing them to naive mice did not raise a statistical significance either (p=0,96) (Figure 7B).

- **CA3**

The frequency of spontaneous spiking events did not differ among the 3 groups in the CA3 region during control aCSF application (one-way ANOVA, F=2,025, p= 0,1594) or during high K^+ aCSF perfusion (one-way ANOVA, F=2,884, p=0,0820) (Figure 7B). Statistical significance was not present either when comparing data from trained vs untrained animals (unpaired t-test, normal aCSF: p= 0,056, high K^+: p=0,06) (Figure 7B).

- **DG**

In the DG, the number of spontaneous spiking events was significantly different among the three groups (one-way ANOVA, F=6,67, p=0,0073). Post-hoc analysis revealed that the number of spontaneous spiking events significantly differed only between the

non-adaptive and the control group (Tukey's test: Adaptive vs Control: p-value=0,17, Non-adaptive vs. Control, p=0,0054)

During High K^+ aCSF perfusion, no significant changes in the numbers of spiking events were among the adaptive, non-adaptive and the control group (one-way ANOVA, F=0,8329, p=0,4509).

Normal vs High K^+ aCSF

Differences between control and High K^+ aCSF were found to be statistically significant only for the spontaneous events recorded in untrained mice slices, where a higher number of spikes were measured in High K+ aCSF. (two-tailed t-test, CA1: p=0,0206, CA3: p= 0,0007, DG: p=0,0099). Changes of the number of spontaneous events among the three HPC subregions were not found to be statistically significant.

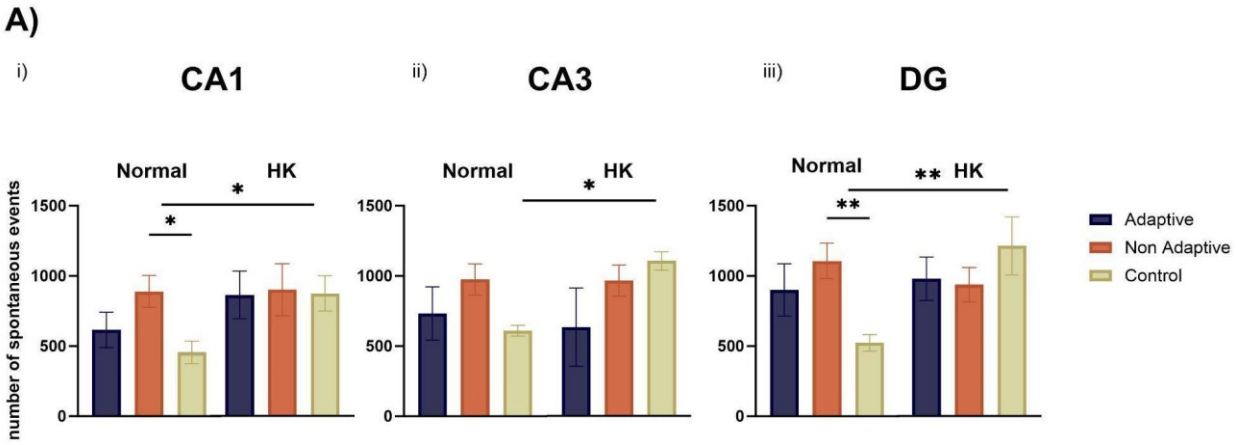


Figure 4. Number of spontaneous spike events in CA1, CA3 and DG hippocampal subfields A) Non adaptive exhibited higher number of spontaneous events in CA1 compared to control(Tukey's test, p=0,54); naive animals exhibited significant increase in number of spikes with High K+ perfusion (two-tailed t-test, p=0,02) ii) No significant differences observed in CA3 regarding spontaneous events numbers in neither trained groups or different aCSF conditions, while untrained mice spontaneous events were increased during High K+ perfusion (p=0,0007), iii) Significantly higher number of spontaneous events of the non-adaptive group in DG compared to controls in normal aCSF; comparisons between aCSF conditions only controls exhibited increase of spontaneous events during High K+ (p=0,009)

6.5.2 Oscillatory activity

Using of the rate of power metric, the oscillatory activity in the range of each band (delta (1–4 Hz), theta (4–7 Hz), alpha (8–12 Hz), beta (13–30 Hz), total gamma (30–150 Hz), gamma (30–80 Hz) and high gamma (80–150 Hz)) was quantified. The electrophysiological recordings were performed in brain slices of the trained & control animals, recording in each one from a different hippocampal subfield (CA1, CA3, DG) and in two aCSF conditions (Control, High K+).

- **CA1**

A tendency for increased frequency rates was apparent when recording from the CA1 region during the perfusion of control aCSF. Significant increases were noted in the rates of Delta (one-way ANOVA, F=2,70, p=0,004, Tukey's test: Adaptive vs Control: p=0,002, Non-adaptive vs Control: p=0,001), Alpha (one-way ANOVA, F=8,55, p=0,0017, post-hoc Tukey's test p-value=0,0043 and 0,072 for Adaptive and Non adaptive vs control respectively) and Beta frequencies (one-way ANOVA, F=9,84, p=0,0008, post-hoc Tukey's test p-value=0,001 and 0,014 for Adaptive and Non adaptive vs control respectively) in both adaptive and non-adaptive animals relative to the controls. Theta, Gamma and High Gamma exhibited tendency for increase, though they did not yield statistically significant values (one-way ANOVA, Theta: F=0,03 , p=0,99, Gamma: F=1,5, p=0,24, High Gamma: F=0,22, p=0,79) (Figure 8A).

Despite the expected further increase of frequency rates when High K+ aCSF was perfused, the whole range of frequencies did not show significant changes among the behavioral groups and the control group (one-way ANOVA, Delta: F=0,74 ,p=0,48, Theta: F=0,17, p=0,84, Alpha: F=0,4, p=0,67, Beta: F=0,15, p=0,86, Gamma: F=0,93, p=0,41 High Gamma: F=1,06, p=0,36) (Figure 8B).

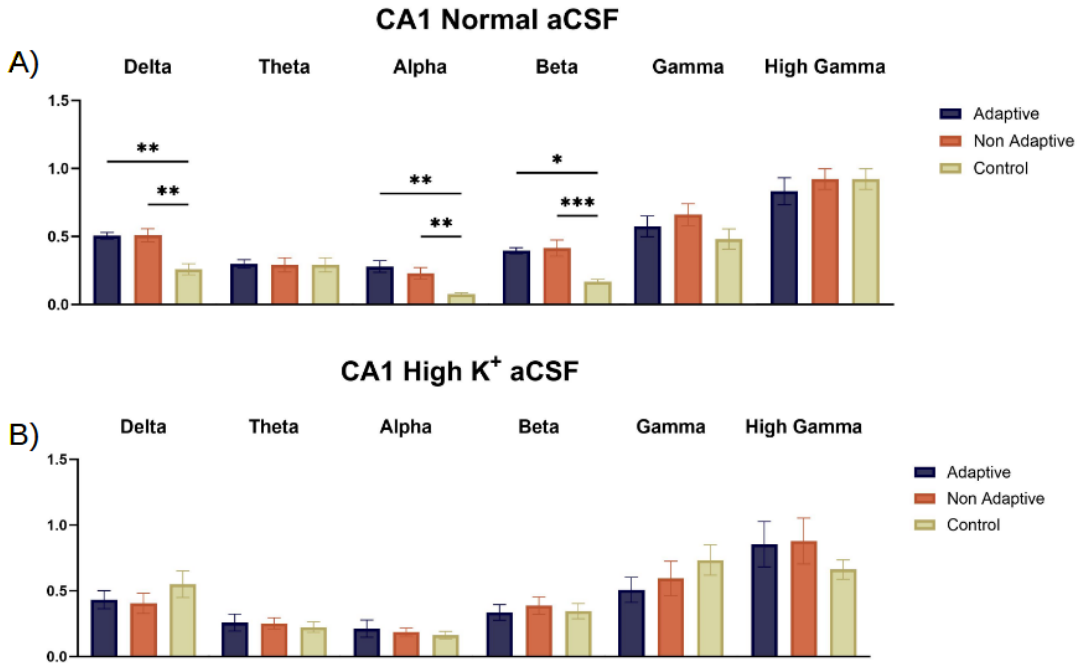


Figure 5. Oscillatory power in CA1 A) Significant increases of Delta (Tukey's Test, p-value=0,002 and 0,0012 for Adaptive and Non adaptive vs Control respectively), Alpha (Tukey's Test, p-value=0,0043 and 0,072 for Adaptive and Non adaptive vs Control respectively), Beta (Tukey's test p-value=0,001 and 0,014 for Adaptive and Non adaptive vs control respectively) frequency rates in CA1 during perfusion of Normal aCSF, B) No significant changes among frequency rates during high K+ aCSF perfusion (one-way ANOVA, Delta: F=0,74 ,p=0,48, Theta: F=0,17, p=0,84, Alpha: F=0,4, p=0,67, Beta: F=0,15, p=0,86, Gamma: F=0,93, p=0,41 High Gamma: F=1,06, p=0,36)

- **CA3**

More evident effects of WMT were observed in the recordings in the CA3 area of the HPC. Significant elevation was observed Delta (one-way ANOVA, F= 6,96, p=0,006, Tukey's Test: p-value=0,005 for Adaptive vs Control), Theta (F=9,07, p=0,001, Tukey's Test: p-value=0,002 and 0,02 for Adaptive and Non-adaptive vs Control respectively), Alpha (F=9,46, p=0,001, Tukey's Test: p-value=0,002 and 0,01 for Adaptive and Non-adaptive vs Control respectively), Beta (F=8,3 , p=0,01, Tukey's Test: p-value=0,007 and 0,01 for Adaptive and Non-adaptive vs Control respectively), Gamma (F=5,3 , p=0,003, Tukey's Test: p-value=0,005 and 0,03 for Adaptive and Non-adaptive vs Control respectively) and High Gamma: (F=4,5, p=0,02, Tukey's Test: p-

value=0,04 for Non-adaptive vs Control) frequency band rates were significantly enhanced during the perfusion of control aCSF. Non-adaptive Alpha (Tukey's Test, p-value=0,15) and Adaptive High Gamma (Tukey's Test: p-value=0,07) frequency rate did not differ from the respective control values (Figure 9A)

Perfusion of HK in CA3 did not appear to have significant effect on the frequency rates, similar as in CA1(one-way ANOVA, Delta: F=0,1725 ,p=0,8428, Theta: F=0,9483, p=0,4041, Alpha: F=1,454, p=0,2574 , Beta: F=0,4227, p=0,6610, High Gamma: F=0,1293, p=0,8794).

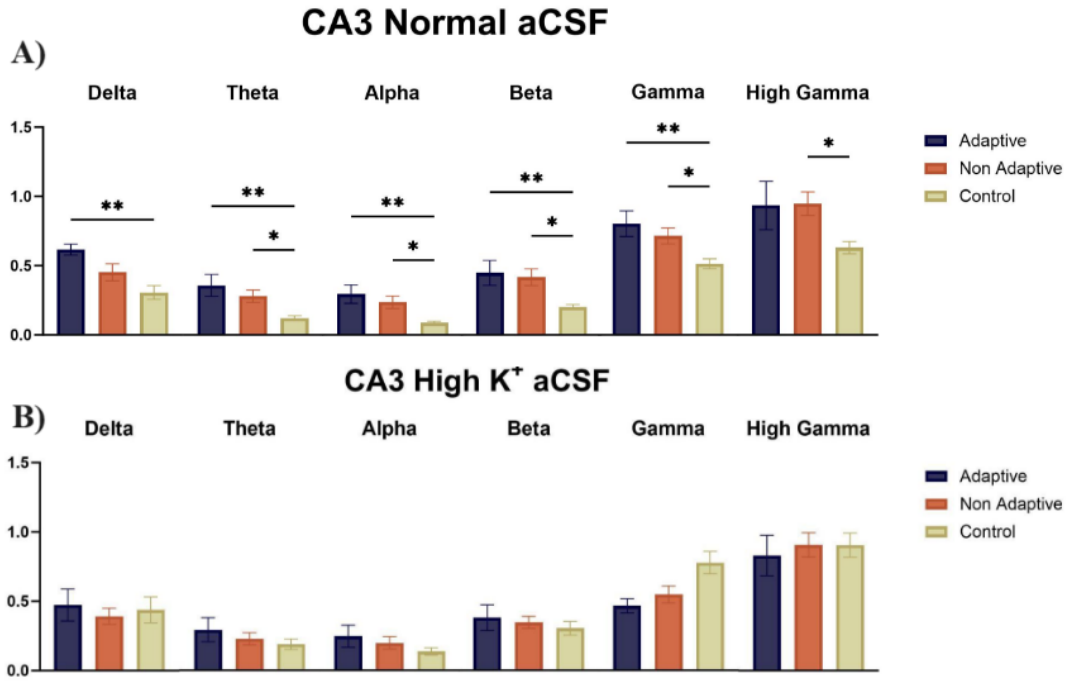


Figure 6. Oscillatory power in CA3 A) Significant increases of Theta, Alpha, Beta, Gamma frequency rates in CA3 of both adaptive and non-adaptive training groups during perfusion of Normal aCSF, B) No significant changes among groups and frequencies during High K+ aCSF perfusion

- **DG**

There was no effect of WM training on oscillatory activity in DG, when perfused with control aCSF (one-way ANOVA, Delta: F=0,09 ,p=0,9, Theta: F=0,7, p=0,5, Alpha: F=0,9, p=0,4 , Beta: F=1,3, p=0,29, Gamma: F=1,04 , p=0,37, High Gamma: F=2,47, p=0,11) (Figure 6A) or High K+ a CSF (one-way ANOVA, Delta: F=0,41 ,p=0,66,

Theta: F=0,2, p=0,81, Alpha: F=0,19, p=0,82 , Beta: F=0,005, p=0,99, Gamma: F=3,65, p=0,051, High Gamma: F=1,06, p=0,36) (Figure 6B).

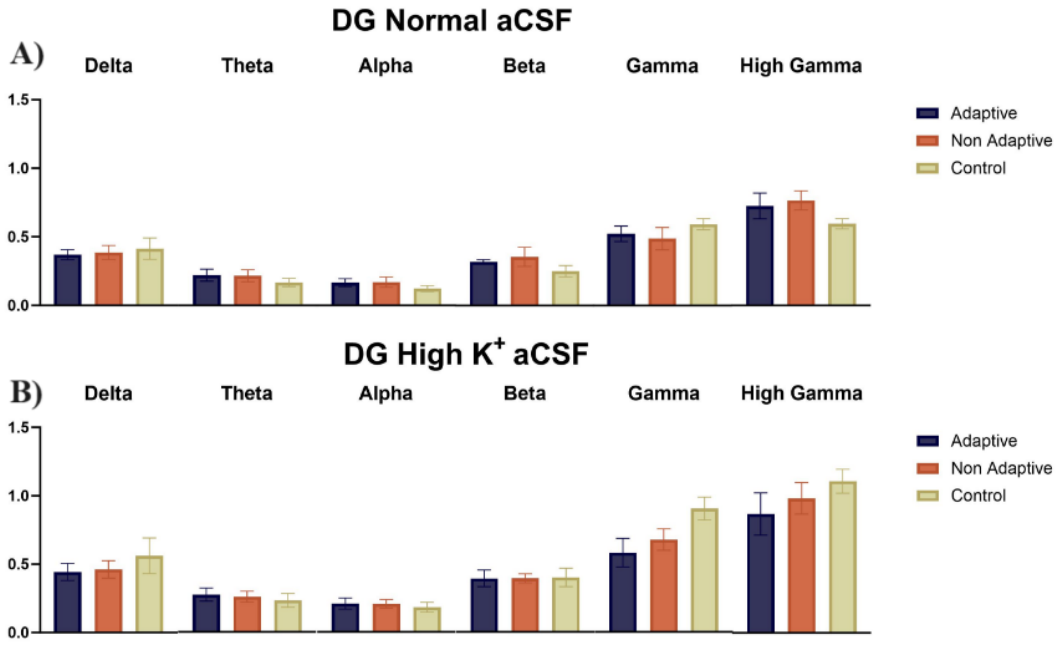


Figure 6. Oscillatory power in DG No significant differences were evident between the adaptive and non-adaptive group oscillations during control aCSF and High K+ aCSF.

Differences in oscillations among HPC subregions (CA1, CA3, DG) were tested in the different behavioral groups and aCSF conditions and no overall changes were detected. The only exception was observed on the average rates of Delta and Beta frequencies of the adaptive group when perfused with control aCSF (one-way ANOVA, F=14,4, p=0,0008). Specifically, Tukey's test revealed that DG delta oscillations were significantly different from those in CA1 and CA3 (p=0,0242 and 0,0007 respectively), whereas the rates of DG Beta oscillations were significantly different from those in CA3 (p=0,0234).

6.6. Effect of WMT on synaptic plasticity

Long Term Potentiation

Two weeks following training, the mice brains were prepared for ex vivo electrophysiological recordings in brain slices in order to record LTP. Both the adaptive and non-adaptive groups showed a significantly enhanced synaptic potentiation following theta-burst stimulation. LTP in the adaptive and non-adaptive mice was

significantly higher relative to the control group both regarding peak amplitude (Repeated Measures ANOVA, F=0,52, p<0,0001) and slope (Repeated Measures ANOVA, F=1,06 , p<0,0001) for 50 minutes after theta-burst stimulation.

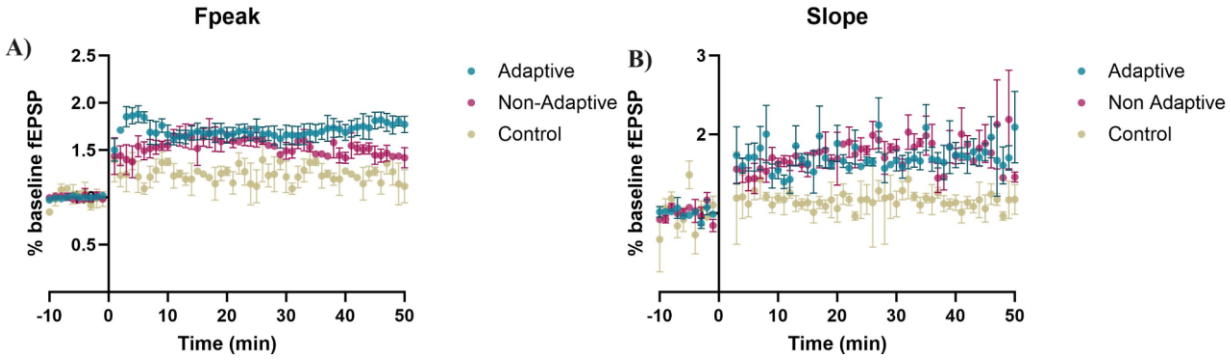


Figure 8. Enhanced LTP of both trained groups 14 days after WM training A) Increased peak amplitude post-theta burst on trained vs naive mice, B) Higher potentiation of adaptive and non-adaptive mice relative to controls following theta-burst.

7. Conclusions

In this study, we investigated the effects of WM training on cognitive flexibility as well as on the spontaneous activity and long-lasting plasticity of hippocampal neurons of adult female mice. Performing a delayed alternation task resulted in decreased anxiety levels and improved cognitive flexibility, when reversal learning was tested on both behavioral groups. Recordings obtained from the CA1, CA3 and DG hippocampal subregions showed increased spontaneous activity in the brain slices of trained mice compared to untrained controls as. Moreover, oscillatory activity of trained mice showed elevated frequency rates in CA1 and CA3, whereas no significant differences were observed in DG. Lastly, the effects of training were evident up to 19 days after completing the task, as mice both in the adaptive and non-adaptive group exhibited enhanced LTP in HPC.

8. Discussion & Future Perspectives

The present set of experiments was carried out in order to gain insights about the effects of WM training in cognitive flexibility, spontaneous activity and neural plasticity in adult female mice. In particular, they provide evidence that WM training enhances cognitive flexibility (the ability to perform different untrained cognitive tasks by flexibly adjusting behavior to new task requirements) by testing reversal learning (the ability to adjust behavioral responses opposed to previously known rules) in female mice. This comes in contrast with a previous study that did not find WM training to result in improved performance in the extradimentional shift of the AST (Attention Switching Test) task, also testing cognitive flexibility (Stavroulaki et al., unpublished data). This may be due to different properties of the tests selected to measure flexible behavior, as AST and reversal learning are based on different aspects of cognitive flexibility. An additional indicator of the effect of training is the reduction of response latency the trained mice had after the T-maze task; this decrease in the time to adjust the focus of attention is a result of increased efficacy of information processing (von Bastian & Oberauer, 2014).

Besides these observed beneficial effects of WM training, the present findings suggest the underlying, training-induced modifications of neural activity. Higher oscillatory power in the hippocampus denote that WM training significantly altered the plasticity. This aligns with previous research of the lab that observed enhanced synaptic properties

of HPC neurons after WM training in male mice (Stavroulaki et al., 2021). However, there are differential effects of WM training dependent on sex. While male mice exhibited enhanced LTP in the PFC and increased fEPSPs in the HPC of male, females exhibited LTP only in HPC (Stavroulaki et.al., unpublished data). This was reflected in the absence of increased spine density in the PFC, in contrast with findings in male mice, which exhibited greater numbers of spines in both PFC and HPC. Complimenting the latter study, the present findings support that this effect of WM training in HPC plasticity lasts at least 2 weeks after training. Further research of differences in the neural circuits affected by WM training in both sexes, will lead to a better understanding of this phenomenon.

Additionally, the fact that WM training seemed to prohibit further increase in neuronal excitability dependent on higher extracellular Potassium levels, may lead to neuroprotective effects in models of epileptic activity. The epileptic discharges of neurons are spontaneous, high frequency neuronal activations with detrimental effects on physical and mental health. The constraint of rises in neural activity that was observed when trained mice brain slices were perfused with high K+ aCSF is encouraging, as patients symptoms and cognitive deficits could be ameliorated by WM training except from pharmacological approaches (Young et al., 2021).

While this study provides some intriguing findings, it is important to identify some of its limitations. Firstly, apart from the active control of the non-adaptive behavioral group, the experiments would lead to more pronounced differences of WM training if there were passive controls during the behavioral procedures. Second, electrophysiological recordings have limited ability to capture the highly dynamic effects of learning during training. Future research utilizing chronic electrode implants would demonstrate the fluctuations of synaptic alternations in the different phases of training.

In summary, the study at hand emphasizes the significance of HPC as a core WM component besides the PFC being the central node. Further research on the effects of WM training will broaden our understanding of brain dynamics and plasticity. WM training and other cognitive training programs should be designed accordingly to utilize to the fullest the multi-level impact it can have on neural physiology and behavior.

9. References

Arski, O. N., Young, J. M., Smith, M. Lou, & Ibrahim, G. M. (2021). The Oscillatory Basis of Working Memory Function and Dysfunction in Epilepsy. *Frontiers in Human Neuroscience*, *14*(January), 1–16. https://doi.org/10.3389/fnhum.2020.612024

Atkinson, R. C., & Shiffrin, R. M. (1968). Human Memory: A Proposed System and its Control Processes. *Psychology of Learning and Motivation - Advances in Research and Theory*, *2*(C), 89–195. https://doi.org/10.1016/S0079-7421(08)60422-3

Baddeley, A. D., & Hitch, G. (1974). Working Memory. *Psychology of Learning and Motivation - Advances in Research and Theory*, *8*(C), 47–89. https://doi.org/10.1016/S0079-7421(08)60452-1

Boran, E., Fedele, T., Klaver, P., Hilfiker, P., Stieglitz, L., Grunwald, T., & Sarnthein, J. (2019). *Persistent hippocampal neural firing and hippocampal-cortical coupling predict verbal working memory load*. Retrieved from https://www.science.org

Bueno-Junior, L. S., & Leite, J. P. (2018). Input convergence, synaptic plasticity and functional coupling across hippocampal-prefrontal-thalamic circuits. *Frontiers in Neural Circuits*, *12*(May), 1–17. https://doi.org/10.3389/fncir.2018.00040

Buzsáki, G., & Watson, B. O. (2012). Brain rhythms and neural syntax: implications for efficient coding of cognitive content and neuropsychiatric disease. *Dialogues in Clinical Neuroscience*, *14*(4), 345–367. https://doi.org/10.31887/DCNS.2012.14.4/GBUZSAKI

Chai, W. J., Abd Hamid, A. I., & Abdullah, J. M. (2018). Working memory from the psychological and neurosciences perspectives: A review. *Frontiers in Psychology*, *9*(MAR), 1–16. https://doi.org/10.3389/fpsyg.2018.00401

Christophel, T. B., Klink, P. C., Spitzer, B., Roelfsema, P. R., & Haynes, J. D. (2017). The Distributed Nature of Working Memory. *Trends in Cognitive Sciences*, *21*(2), 111–124. https://doi.org/10.1016/j.tics.2016.12.007

Chung, A., Jou, C., Grau-Perales, A., Levy, E. R. J., Dvorak, D., Hussain, N., & Fenton, A. A. (2021). Cognitive control persistently enhances hippocampal information processing. In *Nature* (Vol. 600). https://doi.org/10.1038/s41586-021-04070-5

Constantinidis, C., Funahashi, S., Lee, D., Murray, J. D., Qi, X. L., Wang, M., & Arnsten, A. F. T. (2018). Persistent spiking activity underlies working memory. *Journal of Neuroscience*, *38*(32), 7020–7028. https://doi.org/10.1523/JNEUROSCI.2486-17.2018

Constantinidis, C., & Klingberg, T. (2016). The neuroscience of working memory capacity and training. *Nature Reviews Neuroscience*, *17*(7), 438–449. https://doi.org/10.1038/nrn.2016.43

Cowan, N. (1998). Visual and auditory working memory capacity. *Trends in Cognitive Sciences*, *2*(3), 77–78. https://doi.org/10.1016/S1364-6613(98)01144-9

D'Esposito, M. (2007). From cognitive to neural models of working memory. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *362*(1481), 761–772. https://doi.org/10.1098/rstb.2007.2086

D'Esposito, M., & Postle, B. R. (2015). THE COGNITIVE NEUROSCIENCE OF WORKING MEMORY. *Annual Review of Psychology*, *66*, 115. https://doi.org/10.1146/ANNUREV-PSYCH-010814-015031

de Mooij-van Malsen, J. G., Röhrdanz, N., Buschhoff, A.-S., Schiffelholz, T., Sigurdsson, T., & Wulff, P. (2023). Task-specific oscillatory synchronization of

prefrontal cortex, nucleus reuniens, and hippocampus during working memory. *IScience*, *26*(9), 107532. https://doi.org/10.1016/j.isci.2023.107532

Delatour, B., & Gisquet-Verrier, P. (2000). Functional role of rat prelimbic-infralimbic cortices in spatial memory: evidence for their involvement in attention and behavioural flexibility. *Behavioural Brain Research*, *109*(1), 113–128. https://doi.org/10.1016/S0166-4328(99)00168-0

Etter, G., van der Veldt, S., Choi, J., & Williams, S. (2023). Optogenetic frequency scrambling of hippocampal theta oscillations dissociates working memory retrieval from hippocampal spatiotemporal codes. *Nature Communications*, *14*(1). https://doi.org/10.1038/s41467-023-35825-5

Goldman-Rakic, P. S. (1996). Regional and cellular fractionation of working memory. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(24), 13473–13480. https://doi.org/10.1073/pnas.93.24.13473

Gu, B. M., van Rijn, H., & Meck, W. H. (2015). Oscillatory multiplexing of neural population codes for interval timing and working memory. *Neuroscience and Biobehavioral Reviews*, *48*, 160–185. https://doi.org/10.1016/j.neubiorev.2014.10.008

Jaffe, R. J., & Constantinidis, C. (2021). Working Memory: From Neural Activity to the Sentient Mind. *Comprehensive Physiology*, *11*(4), 2547–2587. https://doi.org/10.1002/cphy.c210005

Jung, F., & Carlén, M. (2021). Neuronal oscillations and the mouse prefrontal cortex. *International review of neurobiology*, *158*, 337–372. https://doi.org/10.1016/bs.irn.2020.11.005

Kirkby, L. A., Sack, G. S., Firl, A., & Feller, M. B. (2013). A role for correlated spontaneous activity in the assembly of neural circuits. *Neuron*, *80*(5), 1129–1144. https://doi.org/10.1016/j.neuron.2013.10.030

Kraeuter, A. K., Guest, P. C., & Sarnyai, Z. (2019). The Open Field Test for Measuring Locomotor Activity and Anxiety-Like Behavior. *Methods in Molecular Biology*, *1916*, 99–103. https://doi.org/10.1007/978-1-4939-8994-2_9/COVER

Laubach, M., Amarante, L. M., Swanson, K., & White, S. R. (2018). What, If Anything, Is Rodent Prefrontal Cortex? *ENeuro*, *5*(5), 315–333. https://doi.org/10.1523/ENEURO.0315-18.2018

Levy, R., & Goldman-Rakic, P. S. (1999). Association of Storage and Processing Functions in the Dorsolateral Prefrontal Cortex of the Nonhuman Primate. *Journal of Neuroscience*, *19*(12), 5149–5158. https://doi.org/10.1523/JNEUROSCI.19-12-05149.1999

Light, K. R., Kolata, S., Wass, C., Denman-Brice, A., Zagalsky, R., & Matzel, L. D. (2010). Working Memory Training Promotes General Cognitive Abilities in Genetically Heterogeneous Mice. *Current Biology*, *20*(8), 777–782. https://doi.org/10.1016/j.cub.2010.02.034

Liu, T., Bai, W., Xia, M., & Tian, X. (2018a). Directional hippocampal-prefrontal interactions during working memory. *Behavioural Brain Research*, *338*(September 2017), 1–8. https://doi.org/10.1016/j.bbr.2017.10.003

Liu, T., Bai, W., Xia, M., & Tian, X. (2018b). Directional hippocampal-prefrontal interactions during working memory. *Behavioural Brain Research*, *338*, 1–8. https://doi.org/10.1016/J.BBR.2017.10.003

Liu, Y., Nour, M.M., Schuck, N.W. *et al.* Decoding cognition from spontaneous neural activity. *Nat Rev Neurosci* 23, 204–214 (2022). https://doi.org/10.1038/s41583-022-00570-z

Lonardoni, D., Amin, H., Di Marco, S., Maccione, A., Berdondini, L., & Nieus, T. (2017). Recurrently connected and localized neuronal communities initiate coordinated spontaneous activity in neuronal networks. *PLoS Computational Biology*, *13*(7). https://doi.org/10.1371/JOURNAL.PCBI.1005672

Lundqvist, M., Rose, J., Herman, P., Brincat, S. L. L., Buschman, T. J. J., & Miller, E. K. K. (2016). Gamma and Beta Bursts Underlie Working Memory. *Neuron*, *90*(1), 152–164. https://doi.org/10.1016/j.neuron.2016.02.028

Miller, E. K., Lundqvist, M., & Bastos, A. M. (2018). Working Memory 2.0. *Neuron*, *100*(2), 463–475. https://doi.org/10.1016/j.neuron.2018.09.023

Miller, G. A., Galanter, E., & Pribram, K. H. (1960). *Plans and the structure of behavior.* https://doi.org/10.1037/10039-000

Mongillo, G., Barak, O., & Tsodyks, M. (2008). SynaptiC Theory of Working Memory. *Science*, *319*(5869), 1543–1546. https://doi.org/10.1126/science.1150769

Riley, M. R., & Constantinidis, C. (2015). Role of Prefrontal Persistent Activity in Working Memory. *Frontiers in Systems Neuroscience*, *9*(JAN2016), 181. https://doi.org/10.3389/FNSYS.2015.00181

Rossi, M. A., Hayrapetyan, V. Y., Maimon, B., Mak, K., Shawn Je, H., & Yin, H. H. (2012). Prefrontal cortical mechanisms underlying delayed alternation in mice. *Journal of Neurophysiology*, *108*(4), 1211–1222. https://doi.org/10.1152/jn.01060.2011

Roux, F., & Uhlhaas, P. J. (2014). Working memory and neural oscillations: Alpha-gamma versus theta-gamma codes for distinct WM information? *Trends in Cognitive Sciences*, *18*(1), 16–25. https://doi.org/10.1016/j.tics.2013.10.010

Song, D., Wang, D., Yang, Q., Yan, T., Wang, Z., Yan, Y., … Qing, H. (2020). The lateralization of left hippocampal CA3 during the retrieval of spatial working memory. *Nature Communications*, *11*(1), 1–13. https://doi.org/10.1038/s41467-020-16698-4

Spellman, T., Rigotti, M., Ahmari, S. E., Fusi, S., Gogos, J. A., & Gordon, J. A. (2015). Hippocampal-prefrontal input supports spatial encoding in working memory. *Nature*, *522*(7556), 309–314. https://doi.org/10.1038/nature14445

Stavroulaki, V., Giakoumaki, S. G., & Sidiropoulou, K. (2021). Working memory training effects across the lifespan: Evidence from human and experimental animal studies. *Mechanisms of Ageing and Development*, *194*(December), 111415. https://doi.org/10.1016/j.mad.2020.111415

Stavroulaki, V., Ioakeimidis, V., Konstantoudaki, X., & Sidiropoulou, K. (2021). Enhanced synaptic properties of the prefrontal cortex and hippocampus after learning a spatial working memory task in adult male mice. *Journal of Neuroscience Research*, *99*(7), 1802–1814. https://doi.org/10.1002/jnr.24833

Stokes, M. G. (2015). 'Activity-silent' working memory in prefrontal cortex: a dynamic coding framework. *Trends in Cognitive Sciences*, *19*(7), 394–405. https://doi.org/10.1016/J.TICS.2015.05.004

Szechtman, H., Ahmari, S. E., Beninger, R. J., Eilam, D., Harvey, B. H., Edemann-Callesen, H., & Winter, C. (2017). Obsessive-compulsive disorder: Insights from animal models. *Neuroscience and Biobehavioral Reviews*, *76*(Pt B), 254. https://doi.org/10.1016/J.NEUBIOREV.2016.04.019

Takao, K., & Miyakawa, T. (2006). Light/dark transition test for mice. *Journal of Visualized Experiments : JoVE*, (1). https://doi.org/10.3791/104

Tamura, M., Spellman, T. J., Rosen, A. M., Gogos, J. A., & Gordon, J. A. (2017). Hippocampal-prefrontal theta-gamma coupling during performance of a spatial

working memory task. *Nature Communications*, *8*(1). https://doi.org/10.1038/s41467-017-02108-9

Tozzi, A., Zare, M., & Benasich, A. A. (2016). New perspectives on spontaneous brain activity: Dynamic networks and energy matter. *Frontiers in Human Neuroscience*, *10*(MAY2016), 147690. https://doi.org/10.3389/FNHUM.2016.00247/BIBTEX

von Bastian, C. C., & Oberauer, K. (2014). Effects and mechanisms of working memory training: a review. *Psychological Research*, *78*(6), 803–820. https://doi.org/10.1007/s00426-013-0524-6

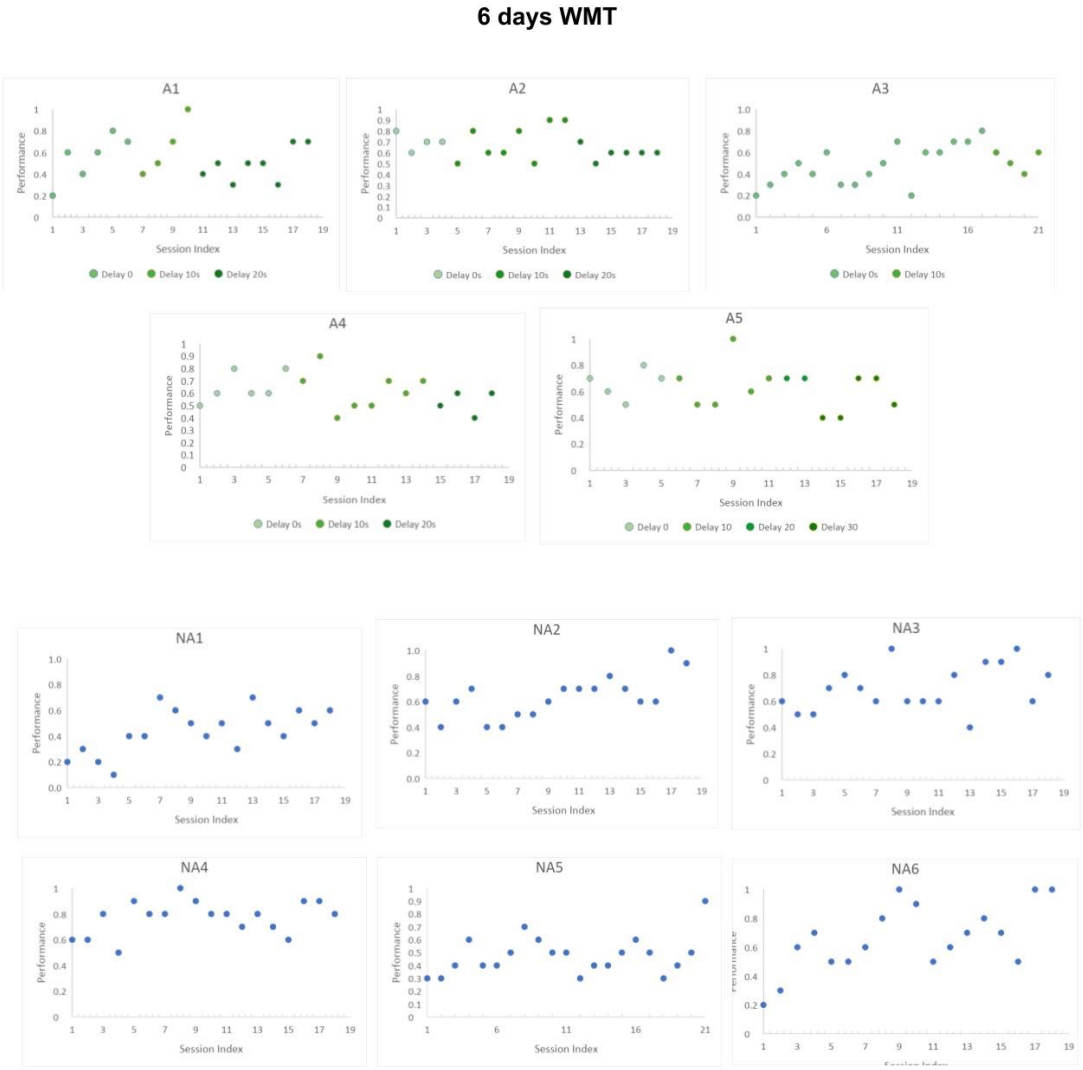
Yangguang Ou, Rachael E Wilson, and S. G. W. (2018). 乳鼠心肌提取 HHS Public Access. *Annu Rev Anal Chem (Palo Alto Calif)*, *11*(1), 509–533. https://doi.org/10.1016/j.nlm.2018.08.023.Disruption

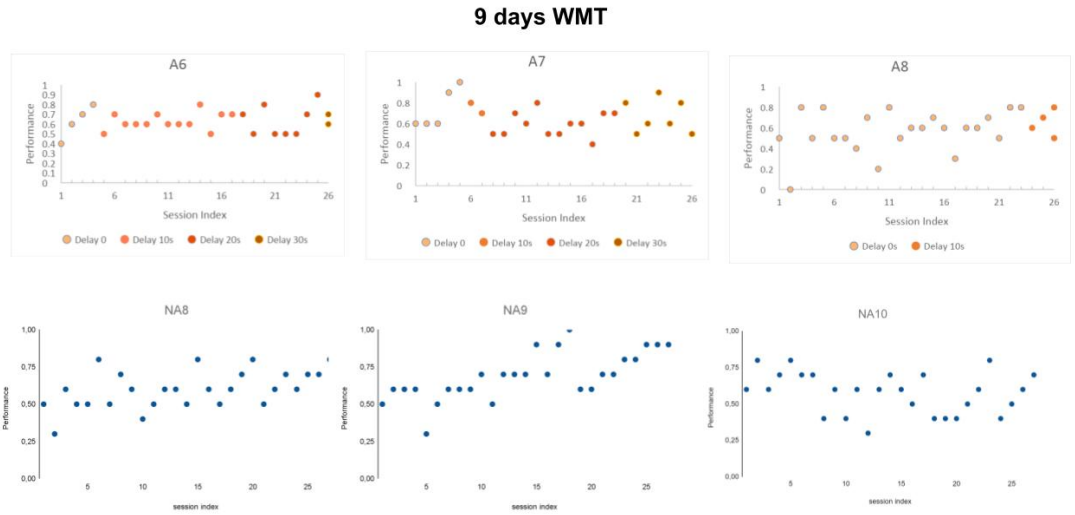
Zhang, W., Guo, L., & Liu, D. (2022a). Concurrent interactions between prefrontal cortex and hippocampus during a spatial working memory task. *Brain Structure and Function*, *227*(5), 1735–1755. https://doi.org/10.1007/s00429-022-02469-y

Zhang, W., Guo, L., & Liu, D. (2022b). Transcerebral information coordination in directional hippocampus-prefrontal cortex network during working memory based on bimodal neural electrical signals. *Cognitive Neurodynamics*, *16*(6), 1409–1425. https://doi.org/10.1007/s11571-022-09792-4

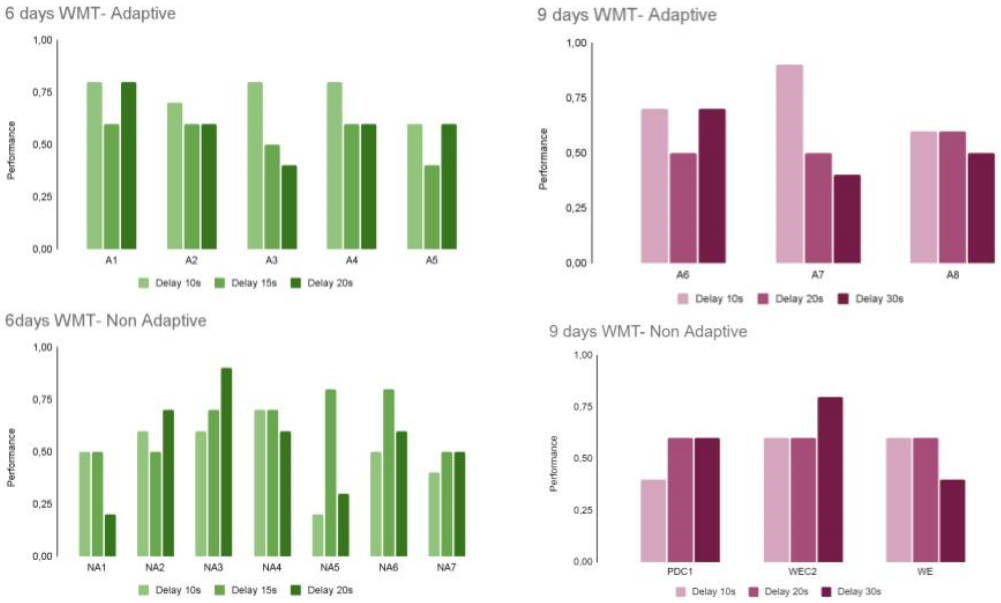
10. Appendix

Working Memory Training Performance

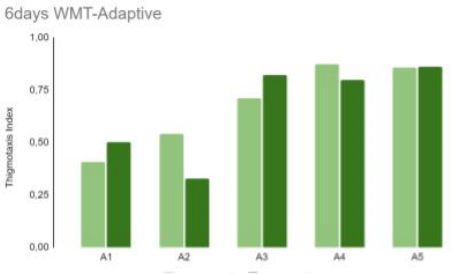


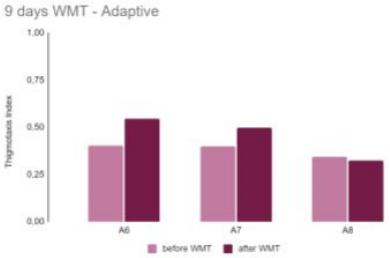


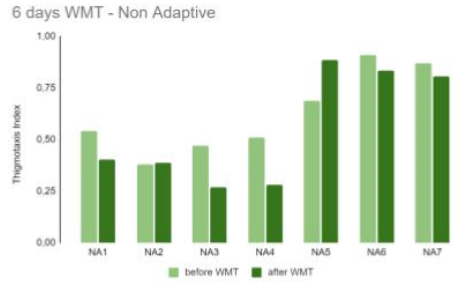
Variable Delays Performance

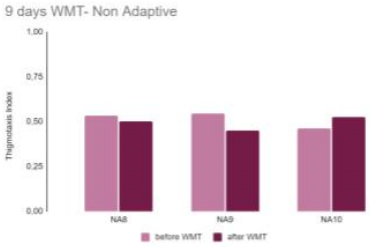


Open Field Test

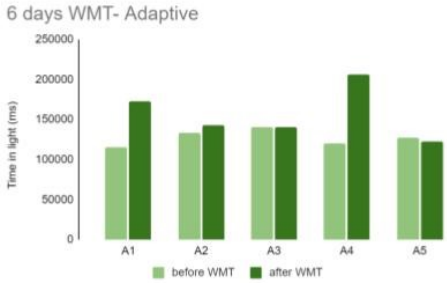


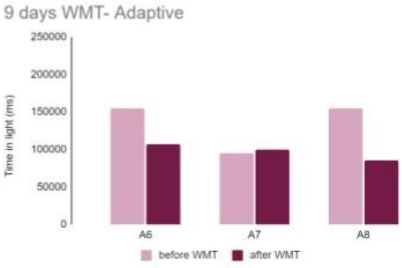


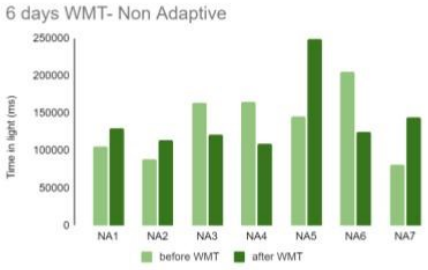


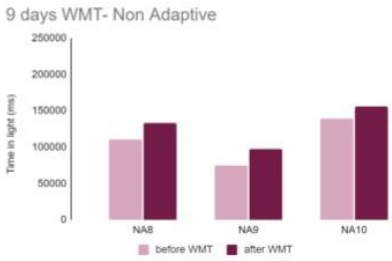


Light-Dark Test

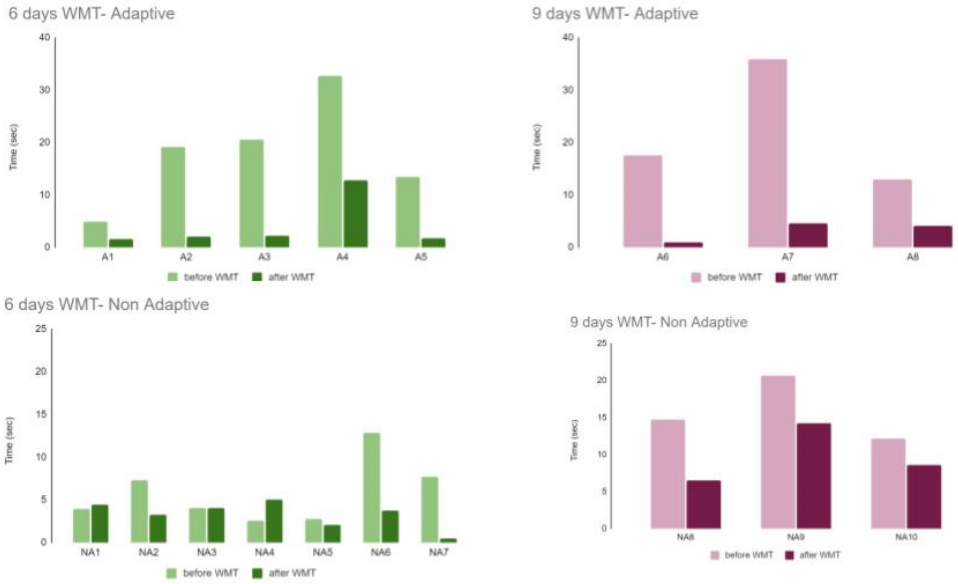




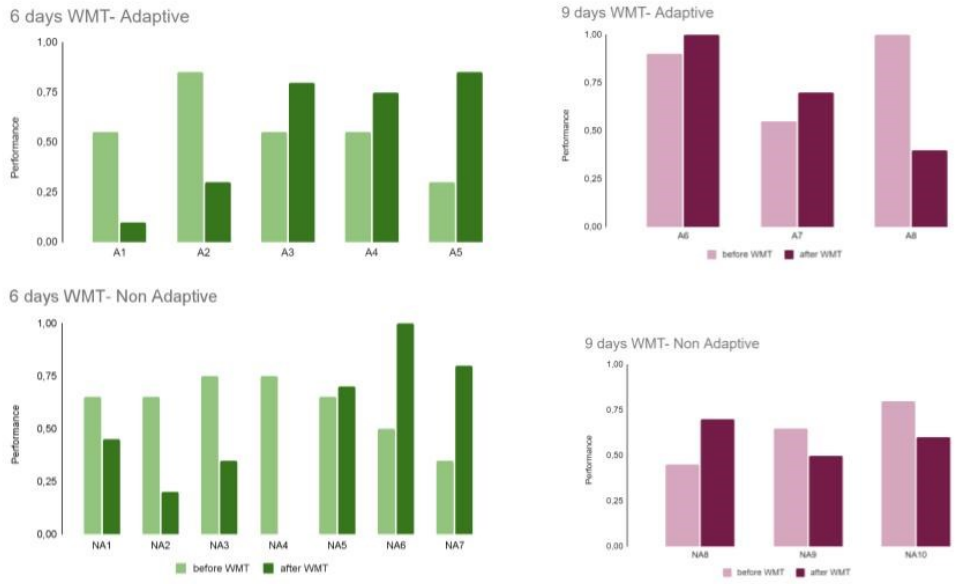




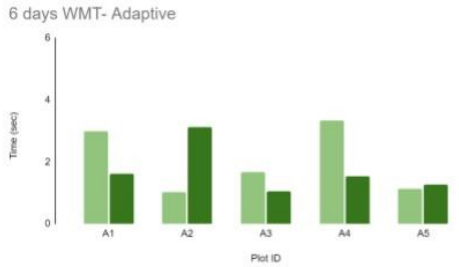
Latency (Time for first entrance in the Light Department)

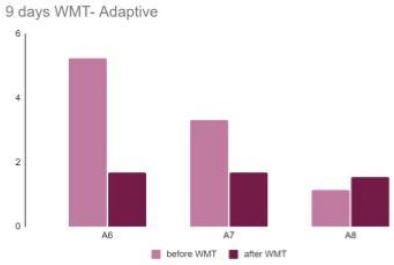


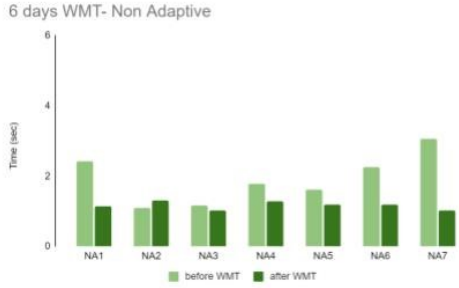
Left-Right Discrimination Performance

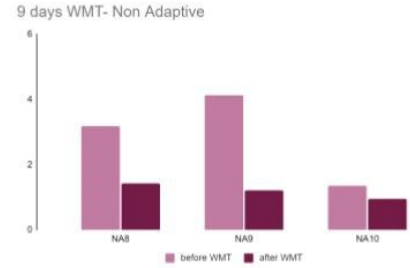


Left-Right Discrimination Latency

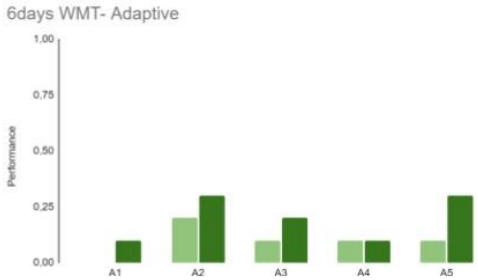


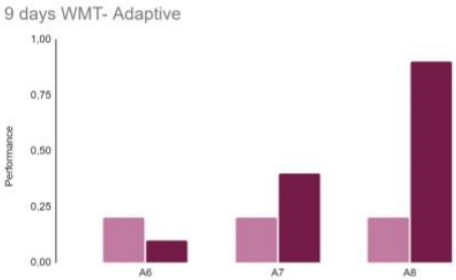


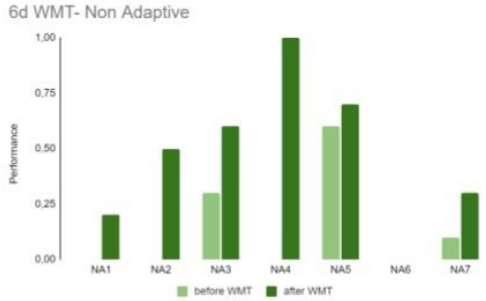


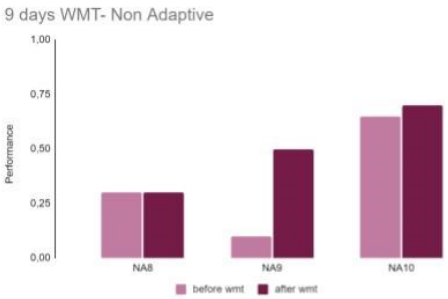


Reversal Learning Performance

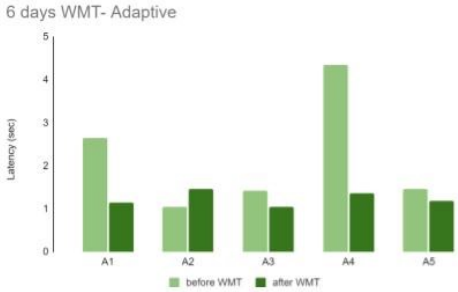


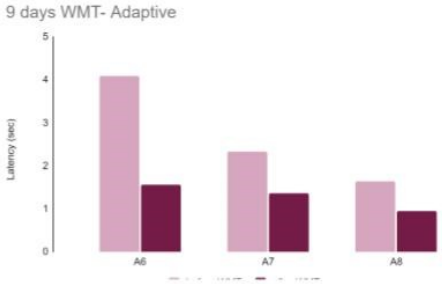


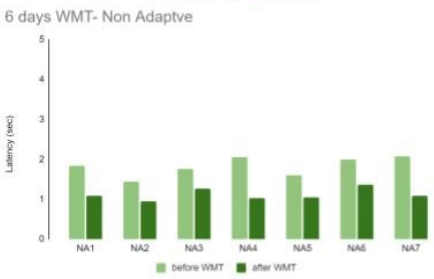


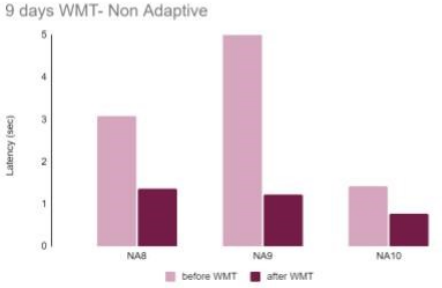


Reversal Learning Latency


6 days WMT- Adaptive
Latency (sec)
0
1
2
3
4
5
A1
A2
A3
A4
A5
before WMT
after WMT


9 days WMT- Adaptive
Latency (sec)
0
1
2
3
4
5
A6
A7
A8


6 days WMT- Non Adaptve
Latency (sec)
0
1
2
3
4
5
NA1
NA2
NA3
NA4
NA5
NA6
NA7
before WMT
after WMT


9 days WMT- Non Adaptive
Latency (sec)
0
1
2
3
4
5
NA8
NA9
NA10
before WMT
after WMT