Title: Effects of working memory training on reference memory, reversal learning and synaptic plasticity in middle-aged male mice

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Abstract

Development of novel pharmaceutical compounds for neuropsychiatric disorders, and particularly for the cognitive symptoms of these disorders, is a significantly slow and expensive process. Alternate therapeutic regimens include cognitive training, a particular form of which is working memory training (WMT). WMT has been suggested to improve several cognitive functions, although the results in the scientific literature are conflicting. However, we still do not understand the neurobiological basis that could explain the beneficial effects of WMT. In our study, we investigated the effects of WMT on reference memory, reversal learning and synaptic plasticity in mice. Specifically, male mice were trained in the delayed alternation task for 9 days and were subsequently tested for retention of left-right discrimination memory and reversal learning. Besides the group that underwent WMT, two control groups were included: an active control group that underwent the alternation procedure in the T-maze but without any delays (non-adaptive) and a passive group that remained in their home cage (naive). The adaptive WMT group performed significantly better in the reversal-learning task compared to both non-adaptive and naïve groups and in the left-right discrimination recall compared to the non-adaptive group only. Electrophysiological recordings in brain slices from the same cohort, 2-3 days following behavioral testing, showed that the adaptive WMT enhanced long-term potentiation (LTP) in the prefrontal cortex (PFC), compared to the non-adaptive or the naive groups. In the hippocampus (HPC), both the adaptive and non-adaptive groups exhibited increased synaptic response to current stimulation compared to the naïve group, but no differences were found in LTP. Our results indicate that WMT affects both behavior and underlying neurophysiological properties, suggesting that this type of training could have beneficial effects in both PFC and hippocampal function.

1. Introduction

Several disorders are characterized by cognitive dysfunctions (Kuperberg and Heckers, 2000) (Gruner and Pittenger, 2017) for which there is currently no effective treatment. Due to several unsuccessful efforts to develop cognitive enhancer drugs, an alternative or additional therapeutic approach could involve cognitive training. Cognitive training approaches require the subjects to perform several mental exercises, designed to engage specific aspects of memory processes. One process of executive function that has received attention in cognitive training approaches is working memory (Constantinidis and Klingberg, 2016), which refers to the ability to hold, maintain and manipulate information on-line for a short period of time (Goldman-Rakic, 1995).

Human studies have shown that working memory training (WMT) not only has an effect on working memory per se, but it can also improve other cognitive abilities such as fluid intelligence (Jaeggi et al., 2008) and attentiveness (Spencer-Smith and Klingberg, 2015), a property known as 'transfer'. Other studies, however, have not identified any improvements (Thompson et al., 2013). In rodents, WMT in the radial arm maze enhanced performance in a variety of other cognitive tasks (Light et al., 2010), and reduced negative behaviors, such as drug-seeking (Boivin et al., 2015).

WMT also modulates neuronal activity in the PFC, the brain area that underlies working memory neuronal activity (Constantinidis and Klingberg, 2016), but also throughout the brain (Buschkuehl et al., 2012), although it is not clear yet how these changes can support the improved cognitive abilities. In addition, WMT modulates dendritic spine density, differentially in different cortical areas, (Comeau et al., 2010), while other types of cognitive training, such as rule learning, enhance axonal plasticity (Johnson et al., 2016).

Despite these initial studies, we still know very little with regards to the effects of

WMT on different behavioral tasks as well as on the underlying cellular mechanisms. In an effort to better understand the behavioral and neurophysiological properties affected by WMT, we investigated the effect of WMT, using the delayed alternation task in mice, on reference memory, reversal learning as well as on synaptic properties and synaptic plasticity in the PFC and the HPC.

2. Materials and Methods

2.1 Animals

Male CV129/B6 mice, aged 7-8 months old (221-278 days old), were used for the experiments. Mice were bred and housed in the IMBB-FORTH facility in groups (3-4 per cage) and were provided with standard mouse chow and water ad libitum, under a 12 h light/dark cycle (light on at 8:00 am) with controlled temperature (24 +/– 1°C). All procedures were performed according to the European Union ethical standards outlined in the Council Directive 2010/63EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes and University of Crete ethical rules.

2.2 Behavioral tasks

The T-maze apparatus used includes a start arm and two goal arms (45X5cm each). The left-right discrimination (LRD) task examines reference memory in mice (Shoji et al., 2012), while reversal learning is a form of cognitive flexibility (Brown and Tait, 2014). The delayed alternation task is a classic task used for the study of working memory and was performed as described before (Konstantoudaki et al., 2017). In summary, mice were initially handled by the experimenter for about a week, and then habituated in the T-maze

apparatus, for 2 days. To make sure that food served as a potent reinforcer, enabling optimal learning of the task, mice had to be food-restricted so that the animals maintained 85-90% of their initial weight. During the second habituation day, the time spent in each arm was calculated in order to establish the arm preference for each mouse. All mice were trained in the LRD task for 2 days. Each mouse, individually, was subjected to a single 20-trial session per day and trained to look for the reward on the arm opposite to the preferred one, as identified in the second habituation day. On the second day, following the 20-trial session, mice were forced to adjust to reversal of the reward for 10 trials.

Following completion of the LRD task and one reversal session, mice were split into three groups. Mice in the naive group remained in their home-cage, while mice in non-adaptive and adaptive groups continued training in the T-maze, this time in the alternation task. 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 non-adaptive group continued to perform the same alternation task for 2 sessions per day, without any delays. Mice in the adaptive group started the delayed alternation procedure, for which delays were introduced starting with 10 seconds and increasing by 10 seconds when the criterion for each delay was achieved, for 9 days. After completion of the delayed alternation procedure, mice were tested for recall of their memory in the LRD task, for 20 sessions, and for their ability to adjust to reversal of the reward, for another 10 sessions.

2.3 Electrophysiological recordings

One to five days following the end of the last behavioral session, mice were prepared for electrophysiological experiment, using *in vitro* slice preparation. The person conducting the electrophysiological experiments was blind to the type of behavioral training performed on the animals. 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). PFC or HPC brain slices (400µm thick) were taken and were transferred to a submerged chamber, which was continuously superfused 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, which continuously superfused 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 PFC upper layers or the stratum radiatum layer of the HPC CA1 region. The platinum/iridium metal microelectrode (Harvard apparatus UK, Cambridge, UK) was also placed within the upper layers of the PFC or HPC, about 300µm away from the recording electrode, and was used to evoke fEPSPs. Responses were amplified using a Dagan BVC-700A amplifier (Dagan Corporation, Minneapolis, MN, USA), 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 consisted of a single square waveform of 100 µsec duration given at intensities of 0.05-0.3 mA generated by a stimulator equipped with a stimulus isolation unit (World Precision Instruments, Inc). The fEPSP amplitude was measured from the minimum value of the synaptic response (4-5 ms following stimulation) compared to the baseline value prior to stimulation. Both parameters were monitored in real-time in every experiment. A stimulus-response curve was then determined using stimulation intensities between 0.05-0.3 mA. For each different intensity level, two traces were acquired and averaged. Baseline stimulation parameters were selected to evoke a response of 1mV. For the LTP experiments in the PFC, baseline responses were acquired for at least 20 minutes, then three 1second tetanic stimuli (100Hz) with an inter-stimulus interval of 20 seconds were applied and finally responses were acquired for at least 50 minutes post-tetanus. For the experiments in the HPC, 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 twice with an inter-stimulus interval of 20 seconds. Synaptic responses were normalized to the average 10 minutes pre-stimulus (tetanus or theta-burst).

2.4 Statistical Analysis

Data analysis was initially performed with Microsoft Excel. One-way, two-way, repeated measures ANOVA or t-tests were performed depending on the experiment. Statistical analysis was performed in IBM SPSS Statistics v.22. Data are presented as mean ± standard error of mean (SEM).

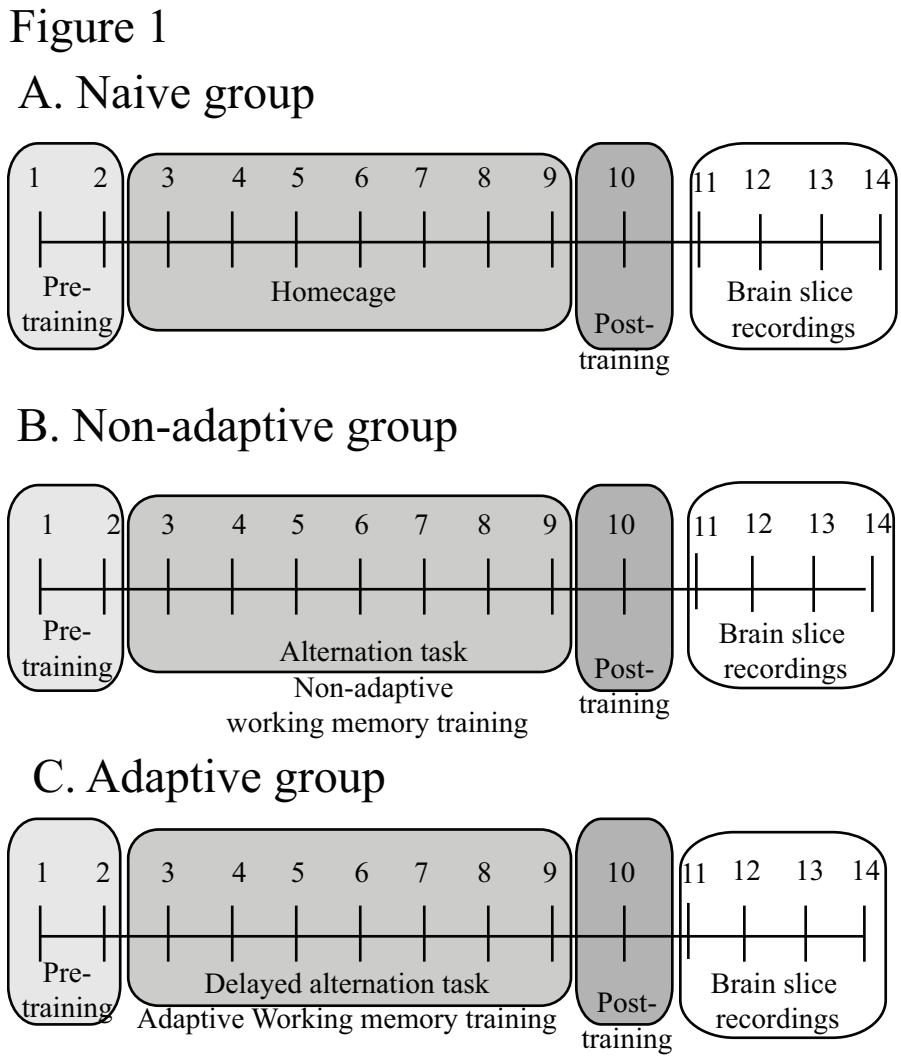

Figure 1

Experimental design of the study, outlining the three different groups used, namely, the naïve, the non-adaptive and the adaptive groups.

3. Results

Thirty-one adult male mice were used in this study. Mice were first trained to acquire the LRD task in the T-maze and were exposed to 10 trials of reversal learning. They subsequently split into three groups (Figure 1): a) the naïve group, in which the mice stayed in their home-cage (passive control), n=9, b) the non-adaptive group in which mice underwent training the alternation task in the T-maze, without the introduction of delays, a group that served as the active control group, n=11, and c) the adaptive group, in which

mice underwent delayed alternation task were trained in a spatial working memory task, n=11 namely the delayed alternation in the T-maze.

3.1 Effects of the delayed alternation task on left-right discrimination and reversal learning

Mice of all three groups learned the LRD task equally well. There were no differences between the two groups in both days of training required to reach the criterion of learning the task (one-way ANOVA, $F_{(2,29)}$=0.25, p=0.5) (Figure 2A). Similarly, there was no difference in the performance of the reversal learning task in all three groups of mice, prior to working memory training (one-way ANOVA, F=0.12, p=0.6) (Figure 2B). Following training in the working memory task, namely the delayed alternation task, mice in the adaptive group performed significantly better compared to mice in the non-adaptive group, but equally well compared to naive mice (one-way ANOVA, $F_{(2,29)}$=2.4, p=0.04) (Figure 2C). The reason that we did not find an effect between these two groups could be explained by the fact that mice in the

Figure 2

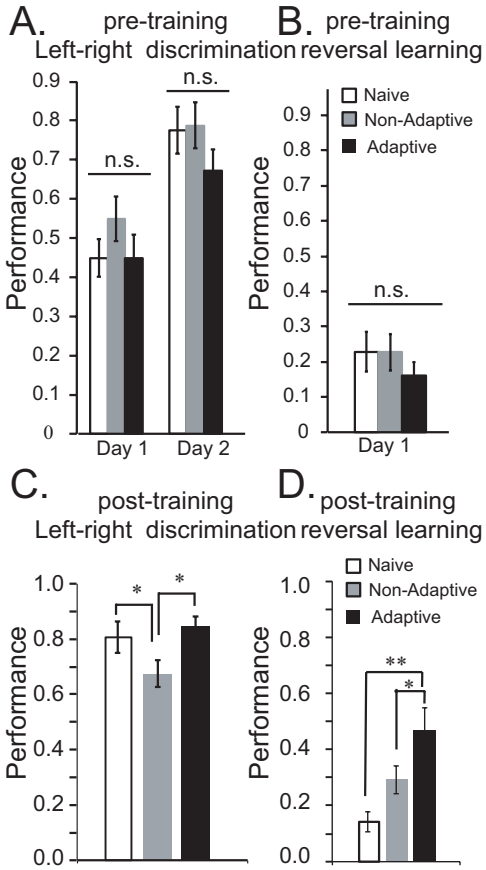

Figure 2

Performance of mice in the left-right discrimination and reversal tasks before and after the delayed alternation task A.Bar graphs showing percent correct in the first and second days of reference memory acquisition in the left-right discrimination task, before WMT. No significant difference among the three groups was identified (one-way ANOVA, $F_{(2,29)}$=1.2, p=0.3) B. Bar graphs showing percent correct in the reversal-learning task, before WMT. No significant difference among the three groups was identified (one-way ANOVA, $F_{(2,29)}$=4.1, p=0.3) C. Bar graphs showing percent correct in the left-right discrimination task after WMT. Significant difference was identified among the three groups (one-way ANOVA, $F_{(2,29)}$=2.4, p=0.4). Post-hoc comparisons identified significant difference between the non-adaptive and the adaptive groups (Tukey test, p=0.03) and the non-adaptive and the naïve groups (Tukey test, p=0.03). D. Bar graphs showing percent correct in the reversal-learning task after WMT. Significant difference was identified among the three groups (one-way ANOVA, $F_{(2,29)}$=6.5, p=0.01). Post-hoc comparisons identified significant difference between the non-adaptive and the adaptive groups (post-hoc Tukey test, p=0.001) as well as the adaptive and naïve groups (post-hoc Tukey test, p=0.005).

naive group did not have to learn to alternate the two arms in the T-maze, which could result in disorientation. With regards to reversal learning, mice in the adaptive group performed significantly better compared to mice in both the non-adaptive and the naive groups (one-way ANOVA, $F_{(2,29)}=3.5$; $p=0.02$) (Figure 2D). There was no significant difference between the naive and the non-adaptive groups.

We next investigated whether performance in the delayed alternation task could correlate with performance in the left-right discrimination and the reversal learning tasks. We plotted the correlation between three different indices of performance in the delayed alternation task, namely, the mean number of sessions required to reach criterion in all delays, the mean percent correct in the delayed alternation task, and the maximum number of consecutive errors and the performance index of the left-right discrimination task and the reversal learning task. We do not find any significant correlations between any of the performance indices of the delayed alternation task and the performance index of the LRD or reversal learning tasks (linear regression analysis, $p>0.05$ for all different combinations) (Figure 3). This suggests that the actual performance in the delayed alternation task does not affect performance in the LRD or reversal learning tasks. Therefore, training in the

Figure 3

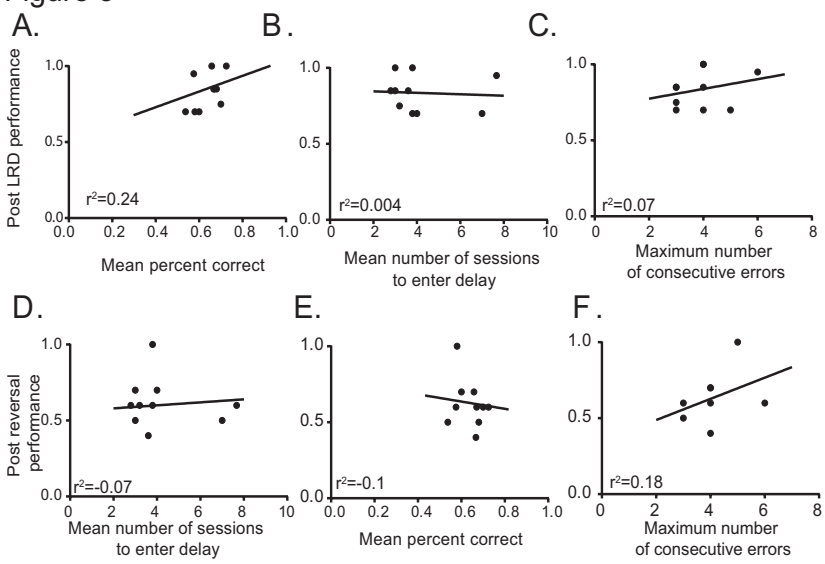

Figure 3
Correlations between performance during WMT and post-WMT performance in LRD and reversal learning tasks.
A-C. Graph showing the correlation between the post-LRD performance (percentage of correct trials) and the average percent correct in the delayed alternation task (A), the average number of trials required to reach criterion in the delayed alternation task (B) and the maximum number of consecutive errors (C). No significant correlations are observed (linear regression analysis, p=0.15, p=0.8, p=0.4 for A, B and C, respectively).
D-F. Graphs showing the correlation between the post-reversal learning performance (percentage of correct trials) and the average percent correct in the delayed alternation task (D), the average number of trials required to reach criterion in the delayed alternation task (E) and the maximum number of consecutive errors (F). No significant correlations are observed (linear regression analysis, p=0.4, p=0.7, p=0.2 for D, E and F, respectively).

delayed alternation tasks improves reversal learning irrespective of performance.

3.2 Effects of working memory training on synaptic transmission and plasticity in the PFC and HPC

Following the behavioral testing, synaptic transmission and synaptic plasticity in the PFC and the HPC was studied, using the brain slice preparation. The PFC supports performance in the delayed alternation and the reversal learning tasks, while the HPC CA1 region mediates performance in the LRD task. Field EPSPs were recorded in PFC layer II while stimulating layer II. There was no significant difference in the fEPSP recorded in response to increasing stimulation in the PFC between the naive, the non-adaptive and the adaptive groups (Figure 4A). This suggests that training in the delayed alternation task does not alter synaptic transmission within the PFC upper layers. On the other hand, the fEPSP recorded in the CA1 region while stimulating the Schaffer collateral axons was increased in both the non-adaptive and adaptive groups of mice, compared to the naive groups (Figure 4B). This suggests that training in the alternation task, with delays or not, which is a spatial working memory task, increases the efficacy of synaptic transmission in the CA1 region of HPC.

Figure 4

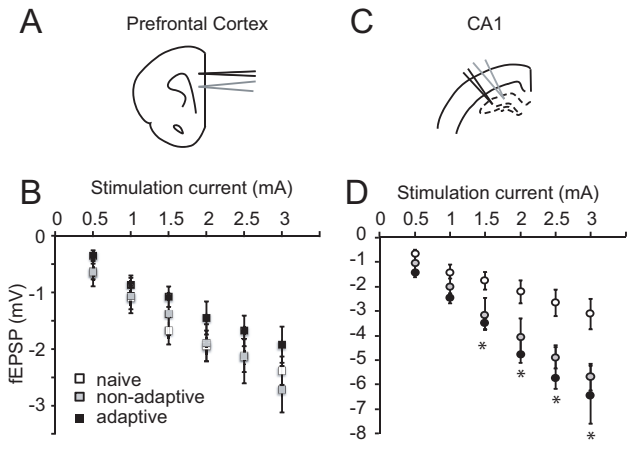

Figure 4

Synaptic transmission in the PFC and the hippocampus, in the naïve, non-adaptive and adaptive groups. A. Electrode positions in the PFC. B. Graph showing that there is no difference in the fEPSP amplitude in response to increasing current stimulation in the PFC (repeated measures ANOVA, $F_{(2,29)}=0.7$, $p=0.5$). C. Electrode positions in the hippocampus. D. Graph showing that there is a significant difference in the fEPSP amplitude in the response to increasing current stimulation in the hippocampus, among the three different groups (repeated measures ANOVA, $F_{(2,29)}=8.5$, $p=0.01$). Post-hoc comparisons show significant differences between the non-adaptive and naïve groups, as well as between the adaptive and naïve groups.

In addition, we examined the induction and maintenance of LTP in both the PFC and HPC. We find that tetanic stimulation resulted in a small, non-significant potentiation of the fEPSP in mice of the naïve and non-adaptive groups. This suggests that LTP in the middle-aged PFC has significantly been decreased compared to the early-adulthood (3-5 months old), when the fEPSP is increased following tetanic stimulation as we have shown in our previous studies (Konstantoudaki et al., 2017; Chalkiadaki et al., 2018). Potentiation of the fEPSP was greater in the adaptive group, and significantly larger compared to the naïve and non-adaptive groups (Figure 5A). These results suggest that LTP has been reduced to non-significant levels at 7 months of age in mice, and that training in a working memory task allows for re-emergence of LTP in PFC layer II synapses of middle-aged mice. In HPC, theta-burst stimulation resulted in fEPSP potentiation in all groups of mice, naïve, non-adaptive and adaptive (Figure 5B). There was no significant difference in the LTP between the three groups.

Figure 5

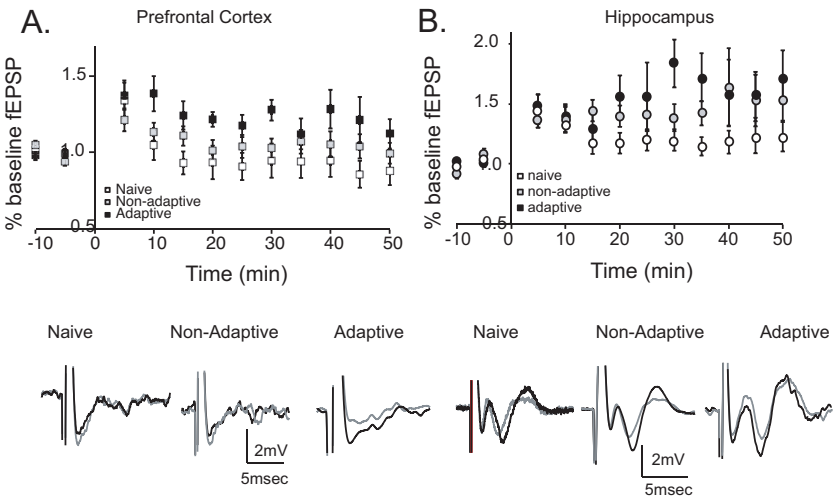

Figure 5

Long-term potentiation in the PFC and the hippocampus of naïve, non-adaptive and adaptive groups. A. Graph (top) and representative traces (bottom) showing the potentiation of the fEPSP following tetanic stimulation in the PFC. There was a significant difference between the three groups (repeated measures ANOVA, $F_{(2,29)}=1.2$, $p=0.03$). Post-hoc comparisons showed that there is a significant difference between the adaptive and naïve (Tukey test, $p=0.02$) and the adaptive and non-adaptive groups (Tukey test, $p=0.03$) for the time points 10min to 50min. In the representative traces, gray trace is before tetanus, black trace is after tetanus. B. Graph (top) and representative traces (bottom) showing the potentiation of the fEPSP following theta-burst stimulation in the hippocampus. There was no significant difference between the three groups (repeated measures ANOVA, $F_{(2,29)}=3.5$, $p=0.2$). In the representative traces, gray trace is before theta-burst, black trace is after theta-burst.

4. Discussion

In this study, we find that training mice in the delayed alternation task significantly improves reversal learning, LTP in the PFC and fEPSP amplitude in the HPC, while also having a beneficial effect on reference memory. Therefore, WMT enhances cognitive flexibility function as well as synaptic plasticity, primarily in the PFC.

4.1 Effects of WMT on PFC function and cognitive flexibility

Our strongest effect of WMT was on reversal learning, a task of cognitive flexibility. A previous study has shown that WMT (using the eight-arm maze) slightly improves several hippocampal-dependent functions, such as fear conditioning and learning the water-maze task, however, it does significantly improve performance in the mouse-adjusted Stroop task (Light et al., 2010), which also examines cognitive flexibility. A recent study also suggests that WMT in humans improves cognitive flexibility, using the intradimentional-extradimentional task (Stavroulaki et al., 2017).

The delayed alternation task is a working memory task that depends on PFC function (Rossi et al., 2012) (Sakurai and Sugimoto, 1985). Persistent activity or short-term plasticity is considered as the cellular correlate of working memory, due to its fast mechanisms (working memory allows us to remember something on-line for a few or several seconds, but not minutes) (Compte, 2006; Riley and Constantinidis, 2015). However, although the actual mechanisms that underlie the on-line maintenance of information in working memory is short-lived, persistent activity (or short-term plasticity) could interact and be facilitated by long-term synaptic plasticity mechanisms (Eriksson et al., 2015). Training to working memory tasks over days increases and strengthens persistent firing (Meyer et al., 2007), which could depend on long-term potentiation processes (Konstantoudaki et al., 2018).

Cognitive flexibility depends on PFC sub-regions, depending on the specific task used. The medial PFC is a brain area that underlies working memory and participates in changes between attentional sets (ref), while orbitofrontal cortex is necessary for reversal learning (Dalton et al., 2016; Izquierdo et al., 2017). Neuronal firing in medial PFC code for the rule of the task, while neuronal firing in orbitofrontal cortex codes for the response outcome (Simon et al., 2015). The PFC is interconnected directly and indirectly, through the mediodorsal nucleus of the thalamus, with the orbitofrontal cortex (Carmichael and Price, 1996; Alcaraz et al., 2016). Therefore, it is likely that enhanced synaptic plasticity in the medial PFC allow for more accurate neuronal firing in the orbitofrontal cortex and better representations of response outcome, therefore, enhanced facilitation of the formation of new rule response outcome.

4.3 Effect of WMT on HPC function

WMT, which primarily depends on the PFC, has been shown to improve reference memory, which is a hippocampal-dependent task (Murray and Ridley, 1999), suggesting that WMT could have a modest transfer, since it improves reference memory, only compared to non-adaptive mice. However, this effect could be due to our experiment design, in which both reference memory testing and WMT took place in the T-maze. Future experiments could explore the effect of WMT on other HPC-dependent behavioral tasks without the use of the T-maze.

In addition, we find that WMT, both in the non-adaptive and the adaptive groups, increases the synaptic response to current stimulation in the HPC. These results could also stem from the fact that the working memory task was a spatial one, therefore, engaging the hippocampus as well. Other spatial tasks have been shown previously to increase neuronal

excitability in the hippocampus for the first 24 hours following training (McKay et al., 2009). The PFC is interconnected with both the orbitofrontal cortex and the hippocampus (Hyman, 2011; Preston and Eichenbaum, 2013), making the possibility for transfer effects from the one to the other plausible.

4.4 Effects of cognitive training

Training in a cognitive task recruits neuronal activity in specific neurons and specific brain areas producing short-term changes in neuronal excitability (McKay et al., 2009) (McKay et al., 2013), which allow for interaction with temporally close distinct training episodes (Cai et al., 2016) and changes in brain areas involved in subsequent learning (Tse et al., 2011). Training in working memory tasks engages neurons in the medial PFC (Baeg et al., 2003; Rossi et al., 2012; Yang and Mailman, 2018), which allows for re-emergence of long-term potentiation capabilities in layer II synapses, as shown in this study. This enhancement in synaptic plasticity could facilitate information transfer from the medial PFC to other interconnected areas, such as the HPC and the orbitofrontal cortex, allowing the enhancement of reversal learning and reference memory. The specific cellular and network events that mediate this transfer are still not known. However, the results of this study highlight the importance of study the effects of learning and/or cognitive training on the underlying brain areas. Such knowledge will allow us to specifically design cognitive training interventions in healthy and patient populations.

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