

## Chemogenetic inhibition of MCH neurons does not alter memory performance in mice

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### ABSTRACT

Memory storage in the brain is one of the most extensively studied subjects in neuroscience. However, due to the highly complex structure of the memory-related systems in the brain, the mystery remains unsolved. Consolidation is one of the most important parts of the memory process, and one that can be affected by numerous neurodegenerative diseases. Hypothalamic melanin-concentrating hormone (MCH) neuronal activity has been of particular interest to researchers in terms of the association between sleep, neurodegenerative diseases, and memory consolidation. We used Pmch-Cre animals to investigate the role of MCH neuronal activity in memory consolidation. In order to observe the differences in memory consolidation, we chemogenetically inhibited MCH neurons using the DREADD method and measured hippocampus-dependent memory performance with a novel object recognition test applicable to early memory impairment in Alzheimer's disease. Our results revealed no significant improvement or worsening with MCH inhibition, suggesting that the role of MCH should now be evaluated in a wider setting.

### 1. Introduction

Memory formation is a curious field of research entailing severe difficulties related to the complexity of the memory process. Minor failures in these mechanisms may have severe consequences. The process of memory encoding and consolidation is strongly associated with sensory stimulation, depending on what is selected for consolidation or forgetting. Several studies have revealed that consolidation and forgetting are two interactive mechanisms with an important role in the shaping of autobiographical memory [1–3]. Studies have already confirmed that consolidation occurs mostly during rest [4–7] and is affected by the concentrations of neurotransmitters such as

acetylcholine, cortisol, and norepinephrine, [8] which modulate memory formation and consolidation in different anatomical regions [6,9].

Melanin-concentrating hormone (MCH) peptides are produced by MCH neurons located in specific hypothalamic regions [10]. Several studies have shown that MCH peptides play a critical role in modulating a wide variety of brain functions, including learning and memory, which may be related to its extensive projections to specific hippocampal regions [11]. Additionally, high levels of MCH-R1 mRNA expression in the CA1 area of the hippocampal formation and cerebral cortex have already been confirmed. These findings were also supported by MCH KO mice studies showing impaired memory performance. Consistent with these findings, animals injected with MCH exhibited increased memory

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performance through the NMDAR-dependent long-term potentiation pathway [10]. However, the hypothalamus's complex anatomy and the cellular heterogeneity of the hypothalamic neuronal populations, along with the wide distributions of their projections, make direct evaluation of MCH-mediated modulation of cognition a challenging matter [11]. However, this difficulty can be overcome using a target-specific approach testing the cognitive role of MCH neurons with specific memory tests attributed to the neuronal circuits involved in MCH-mediated cognition. The novel object recognition (NOR) test is a useful paradigm for assessing the cognitive status of rodents, and one with the advantage of testing task-based spontaneous behavior that does not require any reward or punishment.

It has also been suggested that NOR eliminates possible bias in the interpretation of results deriving from behavioral modifications that could potentially influence memory performance connected with external and internal factors such as drug-related changes, thermoregulation, and anxiety. Due to these various advantages, the NOR test is widely employed for determining recognition memory in different experimental Alzheimer's disease models [12,13].

In order to determine the role of MCH neurons in memory consolidation, we chemogenetically inhibited the MCH neurons in the lateral hypothalamus and zona inserta using the Designer Receptor Exclusively Activated by Designer Drugs (DREADD) method. We then observed the effects of this inhibition on memory consolidation using a NOR memory task with relevance to everyday experiences. In contrast to previous studies [14,15], we mainly focused on consolidating episodic memory, which involves a dynamic interaction between the medial prefrontal cortex and hippocampus [16].

## 2. Method

### 2.1. Animals

Tg (*Pmch-cre*)1Lowl/J (Jackson Labs Stock 014099) mice and C57BL/6 (Jackson Labs Stock 000664) were backcrossed for breeding. PCR performed genotyping before the experiments. The animals were housed at 22–24 °C with a 12 h light/dark cycle. They had ad libitum access to food and water. All experiments were approved by Istanbul Medipol University Animal Research Local Ethics Committee (Decree No:62). 6–10 weeks old male mice were used for all experiments and during stereotaxic surgeries and before the intraperitoneal injections, animals were anesthetized with 1 % isoflurane to avoid animal suffering.

### 2.2. Recombinant adeno associated viral vectors (rAAV) and virus production

Recombinant adeno-associated virus production was performed as previously described (Mathews *et al.*, 2002). Cre-dependent rAAV plasmids used in this study were gifts from Bryan Roth (Addgene plasmid #50461; <http://n2t.net/addgene:50461>; RRID: Addgene 50461) and Edward Boyden (Add gene plasmid #28304; <http://n2t.net/addgene:28304>; RRID: Addgene\_28304).

### 2.3. Stereotaxic injections

Injections were performed for two groups with AAV2/8-EF1a-DIO-hM4D(Gi)-mCherry and AAV2/8-FLEX-GFP viral injections. Animals were anaesthetized with 1 % isoflurane (30 % O<sub>2</sub>, 70 % N<sub>2</sub>O) and placed on a stereotaxic instrument (David Kopf Instruments, Tujunga-CA). After ensuring that the head was placed, the scalp was incised, and the skull was drilled. 500 nl of the virus was injected into both hemispheres for the targeted coordinates using pulled glass pipettes (Drummond Scientific, Wiretrol, Broomall-PA). Injection coordinates were AP: – 1.4/+ 1.4 mm, ML: – 1.5 mm, DV: – 4.7 mm. Paxinos and Franklin's mouse brain atlas is used as a guide. Operated mice were housed in groups of 3–4 animals. We tried to provide half of the cage with control,

the other half as experimental injections. Operation times were similar within the groups. Animals were housed in a post-operation room for at least two weeks before the behavioral analysis for recovery.

### 2.4. Electrophysiology

The mouse was deeply anaesthetized with isoflurane, and the brain was extracted. Extracted brain was sectioned to obtain 300 µm coronal sections in recently prepared cutting solution (234 g/mM sucrose, 28 g/mM NaHCO<sub>3</sub>, 2,5 KCl, 7 g/mM dextrose, 7 g/mM MgCl<sub>2</sub>, 0,5 g/mM CaCl<sub>2</sub>, 1 g/mM sodium ascorbate, 3 g/mM sodium pyruvate, 1,25 mM NaH<sub>2</sub>PO<sub>4</sub>). The solution was aerated with 95 % O<sub>2</sub> and 5 % CO<sub>2</sub> containing gas mixture. Following sectioning, the brain tissue was transferred to artificial cerebrospinal fluid (119 g/mM NaCl, 25 g/mM NaHCO<sub>3</sub>, 7 g/mM dextrose, 2,5 g/mM KCl, 7 g/mM MgCl<sub>2</sub>, 0,5 g/mM CaCl<sub>2</sub>, 1 g/mM sodium ascorbate, 3 g/mM sodium pyruvate, 1,25 mM NaH<sub>2</sub>PO<sub>4</sub>). The brain slices were incubated in this solution for 30 min at room temperature. Infected neurons were detected with a fluorescence microscope attached to the patch-clamp equipment, and cell-attached recordings were measured for neuronal activity. The intracellular solution for the recordings was the artificial cerebrospinal fluid solution. The resistance of the tips was 4–5 MΩ. 3.5 mM clozapine solution was added during recording, and the subsequent effect was observed.

### 2.5. Novel object recognition test

Experiments were held in a white plexiglass apparatus containing four compartments. Each compartment had 40×40×40 dimensions. The experiment was performed as previously described [17] and modified according to Prince *et al.*'s study [18]. The test was performed on three days. On day 1, animals were habituated by allowing to explore the apparatus for 6 min. On day 2, animals were exposed to two identical objects located on the centerline of the apparatus for 6 min, and this step was repeated three times. On day 3, one of the objects was replaced with another one with a different shape but similar size again for 6 min. The exploration time of the objects on day 3 is manually recorded, and the difference between the exploration time of the novel object and the old object was used as a memory performance score. On each day, a maximum of four mice were analyzed. Experiment and control groups were tested simultaneously to discard environmental effects (For example, 2 MCH inhibited and 2 control mice per day). The experiment group had 10 mice and the control group had 9 mice in total. Ethanol was used between trials to clean the apparatus to diminish odor cues. The experiment was held in two sections. In the first set, all experiment and control mice were intraperitoneally saline-injected on day 2. After the completion of the saline set, all experiment and control mice were injected with clozapine. Therefore, each mouse encountered both saline and clozapine injections. The order of the objects was randomized between saline and clozapine conditions. The performer was blind to the experimental groups during behavioral testing and scoring. Clozapine was injected one hour after the training because consolidation takes place within one hour and four hours after encoding, and clozapine exerts its effects after approximately 30 min [19]. Study and control groups were tested simultaneously to prevent environmental complications during testing. Exploratory behavior was determined manually by observing the sniffing and touching behavior of the mice by using the guidelines previously described [20]. The results were analyzed with a two-tailed paired Student's t-test.

### 2.6. Immunohistochemistry and imaging

At the end of the behavioral experiments, training with identical objects was repeated with new objects, and animals were sacrificed by cardiac perfusion with 4 % paraformaldehyde with phosphate-buffered solution (0.1 M). Brains were removed and held in first 4 % paraformaldehyde solution for 4 h and then 30 % sucrose solution until the

brains sunk. Brain tissue was sectioned with a vibratome with 80  $\mu\text{m}$  thickness (Leica VT1000S). They were immunohistochemically labeled with polyclonal MCH antibody (Phoenix Pharmaceuticals H-070–47).

### 2.7. Statistical analysis

The results in the bar charts were represented as means of  $\pm$  SEM. The student's t-test has been used to compare memory performances of chemogenetically inhibited animals and control groups with Microsoft Excel (Version 16.16.19). The exact values of the memory performances of the animals were depicted in bar charts combined with line charts. Memory performances were calculated as a discrimination index equation as it is described in previous literature [20].

## 3. Results

### 3.1. Confirmation of MCH neuron inhibition

To investigate the role of MCH neurons in memory consolidation, we inhibited MCH neurons with DREADD, a chemogenetic manipulation method. Originally, clozapine-N-oxide was used as a secondary agent in the DREADD system; based on recent findings showing that there is no substantial difference between the application of clozapine-N-oxide and clozapine in a similar experimental design [21,22] Cre dependent Tg (*Pmch-cre*)1Low1/J and AAV2/8-EF1a-DIO-hM4D (Gi)-mCherry injected mouse was analyzed for cell-attached patch-clamp recording after two weeks of recovery. Injected MCH neurons were determined by mCherry fluorescence packaged in the virus (Fig. 1a). During spontaneous neuron activity, recording 3.5 mM clozapine is perfused into the bath solution, and within 1–2 min, a decrease in spontaneous activity is observed

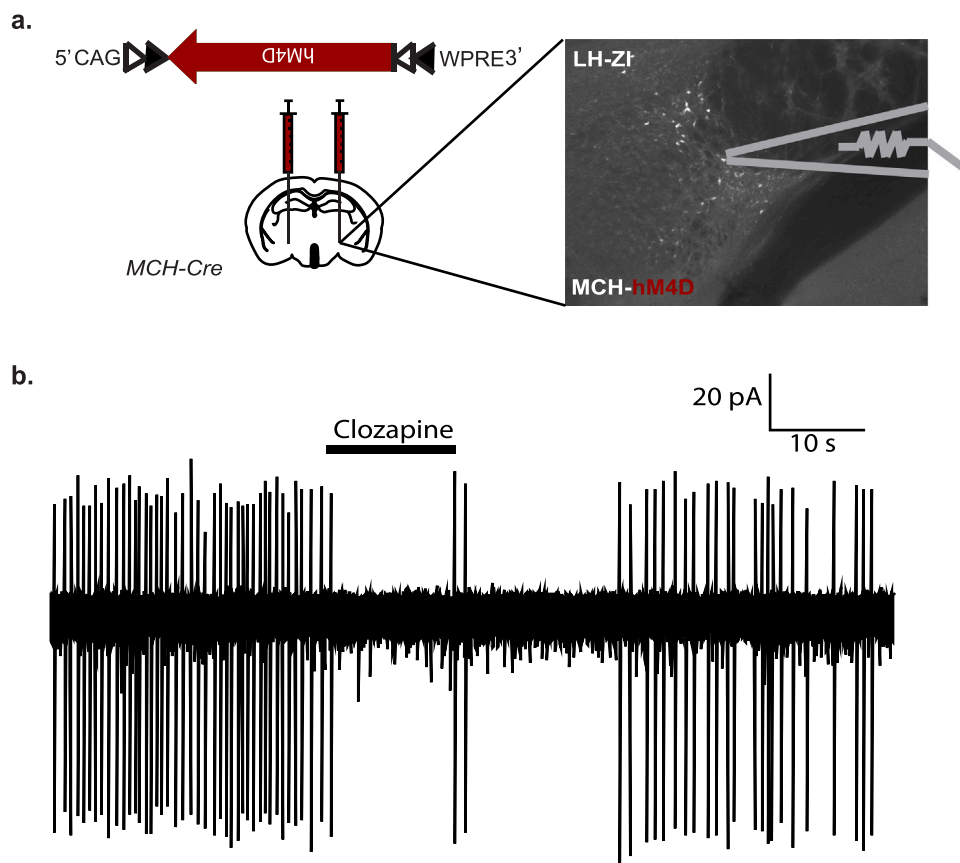
(Fig. 1a-b). Upon perfusion, the activity was regained (Fig. 1b).

### 3.2. The effect of MCH inhibition on memory consolidation

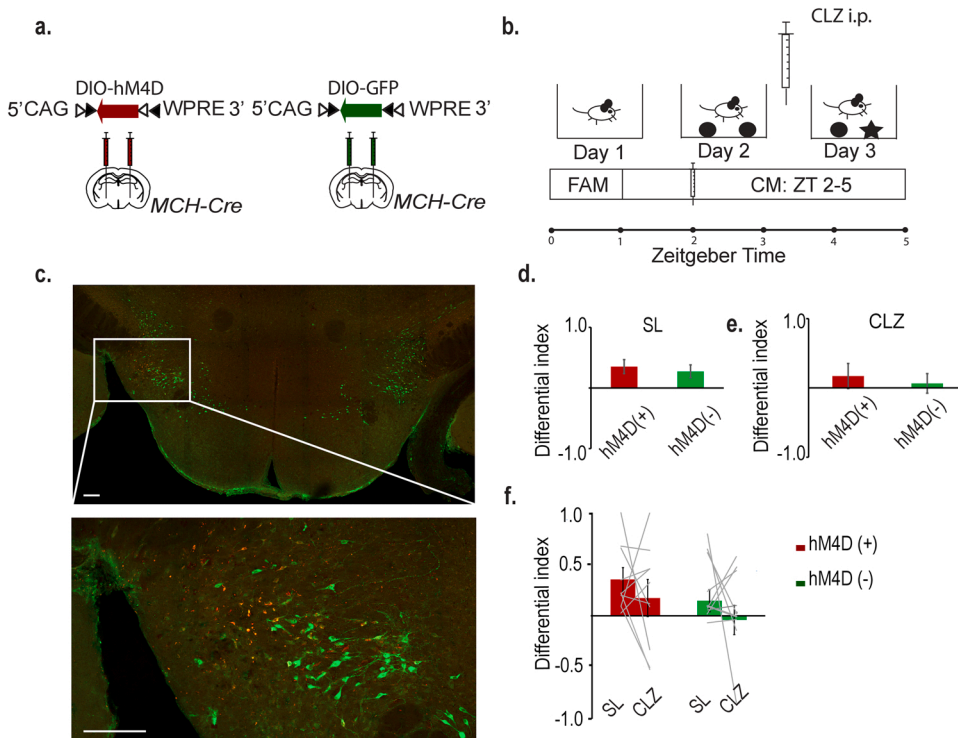
One group was injected with AAV2/8-EF1a-DIO-hM4D(Gi)-mCherry as the experiment group, and another was injected with AAV2/8-FLEX-GFP as a control during novel object recognition test (Fig. 2a). After the recovery period, the animals were habituated to the apparatus on day 1. On day 2, one hour after the trial phase, clozapine was injected intraperitoneally to inhibit the MCH neurons within the period required for memory consolidation [18,19] (Fig. 2b). Saline-injected animals were used as controls. We used clozapine in the concentration of 0.01 mg/kg. The next day, we performed the test phase, and video recorded the exploratory behavior of the animals. Exploration (sniffing and touching) time was manually measured to be able to score the exploratory behavior more precisely. Immunohistochemical analysis confirmed infection sites after the behavioral analysis termination (Fig. 2c). The differential index between novel object exploration and old object exploration gave the memory performance score as described [22]. According to the statistical analysis, there was no difference between MCH inhibited animals ( $p = .39$ , Student's T-Test) and control groups ( $p = .38$ , Student's T-Test) in terms of memory performance (Fig. 2d). At the end of the behavioral tests, mice were sacrificed with new objects after one last trial phase. Immunohistochemistry staining was performed with anti-MCH antibody and MCH stained neurons.

## 4. Discussion

This study investigated the effect of inhibited MCH neurons on memory performance using a NOR test and a chemogenetic approach for



**Fig. 1.** Clozapine is effective in DREADD system a. Schematic representation of stereotaxic injections to the lateral hypothalamus and zona inserta (left), AAV2/1-EF1a-DIO-hM4D(Gi)-mCherry expressing neurons (right) b. Spontaneous neuron activity before and after clozapine perfusion. CAG: cytomegalovirus early enhancer/chicken  $\beta$ -actin promoter, WPRE: woodchuck hepatitis virus, L.H.: lateral hypothalamus, Z.I.: zona inserta.



**Fig. 2.** The effect of MCH inhibition on memory consolidation. a. Schematic representation of viral injections to the lateral hypothalamus and zona inserta with hM4D and GFP. b. Experimental design of novel object recognition test. c. hM4D MCH neurons immunohistochemically labeled with MCH antibody. Bars: 100  $\mu$ m. d. Differential index of the exploratory behavior between hM4D(+) (red) ( $n = 10$ ) and hM4D(-) (green) ( $n = 9$ ) mice in saline condition. e. Differential index of the exploratory behavior between hM4D(+) (red) and hM4D(-) (green) neurons in clozapine condition. f. Paired comparison of the memory performances of hM4D(+) and hM4D(-) mice. CAG: cytomegalovirus early enhancer/chicken  $\beta$  actin promoter, WPRE: woodchuck hepatitis virus, CLZ: clozapine, CM: chemogenetic manipulation, ZT: zeitgeber time, FAM: familiarization.

the inhibition of MCH neurons. Previous research involving the administration of the MCH peptide into the amygdala or hippocampus has yielded interesting results, suggesting a direct role of MCH peptide in increasing memory performance. For example, Monzon et al. showed that microinfusion of MCH peptide into the hippocampus or amygdala (immediately after training) produces an augmentation of memory performance on the inhibitory avoidance test. However, infusion into the entorhinal cortex kept the behavioral outcomes largely intact [23]. In further research evaluating the time-correlated effects of MCH, those authors observed that memory performances improved only when the hippocampal infusions were performed four hours after training. MCH peptide administration has recently been shown to exhibit therapeutic effects in mouse models of memory impairment and Alzheimer's disease [24]. These findings suggest that MCH neuronal activation may play an essential role in increasing memory consolidation, fitting well with recent data in which short-term memory performance decreased when MCH neurons were ablated in adulthood. In their novel study, le Barillier et al. [15] found that MCH/ataxin3 ablated mice exhibited impaired short-term plasticity associated with decreased specific memory performance. However, those authors were unable to completely exclude the potential impact on some essential physiological differences of lower overall body weights due to complete dysfunction of MCH neurons in MCH/ataxin3 mice [15]. Moreover, it was impossible to determine a specific time window for the MCH neurons to become effective in memory consolidation, since the MCH neuronal inhibition was not temporary in this ablation model.

In contrast, Izawa et al. recently showed that optogenetic and chemogenetic inhibition and activation of MCH neurons improved and impaired, respectively, memory performance [25]. In evaluating hippocampus-dependent memories, those authors also performed MCH neuron ablation with tet-off system-controlled diphtheria toxin A expression and confirmed the role of MCH neurons in memory formation. However, in assessing the underlying mechanisms of the action of MCH neurons, they observed no increased frequency of firing following MCH stimulation, although inhibitory presynaptic current amplitude and frequency were altered. This dilemma continued to manifest itself in recent studies showing that hypothalamic MCH neurons play a

functional role in object novelty. In contrast to other studies, Kosse et al. performed selective optogenetic silencing during the initial object memory acquisition, preventing future object recognition by means of MCH receptor-dependent pathways [26]. It should also be remembered that despite a variety of study-specific methodological differences, one common barrier shared by all previous MCH studies may be the wide range of cortical and subcortical projections of MCH neurons [15,24,25,27].

The results of the present study show that inhibition of MCH neurons does not cause a significant difference in memory performance when this is measured using a NOR test. In this research we used a memory performance test with acute inhibition of MCH neurons which was neither reward nor survival-based. Additionally, we applied clozapine in a 3-hour window specified as the specific window for consolidation [24]. The present test was less stressful and purely dependent on the animals' motivation.

Although preliminary in nature, and in contrast to earlier research, this is one of the few studies to test the role of MCH neurons during memory consolidation by means of an acute approach to the inhibition of the MCH neurons. Although the present study yielded valuable data concerning the functionality of MCH neurons in a mixed arousal state, certain limitations should also be borne in mind. First, we could not evaluate the sleep stages and not completely exclude the potential effect of sleep modulation on the behavioral impact of MCH modulation. However, our conditions replicate real-life conditions where the effect of physiological changes present during sleep and arousal states are evaluated together without any external stimulation that could bias cognitive outcomes related to normal sleep physiology. It should also not be forgotten that factors such as the heterogeneity of MCH neurons as well as their variable infection percentages might affect the study results. We therefore suggest that the behavioral analysis might usefully be replicated with a double transgenic line (Tg (*Pmch-cre*)<sup>1</sup>Lowl/J::R26-LSL-hM4Di-DREADD) in order to eliminate the possible bias in the current study. Our findings are also significant in terms of Alzheimer's disease-type impairment of early episodic memory and its potential connection with sleep in the disease pathogenesis. In that context, several cerebral structures are involved in the performance of the NOR test, including the

hippocampus and entorhinal and perirhinal cortices, shown to be involved in the MCH circuit and to be damaged in Alzheimer's disease [13,28–32]. It would also be interesting to evaluate the role of MCH neurons in the pathogenesis of Alzheimer's disease by analyzing the causal relationship between an inhibited MCH population, sleep/wake state acquisition, and memory consolidation in Alzheimer's disease transgenic mice models.

#### CRedit authorship contribution statement

**Ozlem Mutlu Burnaz:** Conceived and designed the experiments, performed the experiments, analyzed the data, prepared the figures and/or tables and drafted the work or revised it critically for important content. **Burak Yulug:** Conceived and designed the experiments, analyzed the data, wrote the manuscript, supervised the whole study protocol and revised the manuscript critically for important content, writing–review & editing. **Merve Oncul:** Performed the electrophysiological analysis, revised the manuscript critically for important content. **Esref Celik:** Participated in histological experiments, revised the manuscript critically for important content. **Nilufer Sayar Atasoy:** Provided technical assistance for the viral delivery and mice genotyping, revised the manuscript critically for important content. **Seyda Cankaya:** Statistical analysis. **Lutfu Hanoğlu:** Conceived and designed the experiments, drafted the work, or revised it critically for important contents. **Halil Aziz Velioglu:** Analyzed the data, supervised the whole study protocol, and revised the manuscript critically for important content, writing–review & editing.

#### Conflict of interest statement

None.

#### Data Availability

The data that has been used is confidential.

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