Original Article

Involvement of the Orexin 1 and 2 Receptors in Nucleus Incertus (NI) on Modulation of Spatial Reference Memory in the Morris Water Maze

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ABSTRACT

The nucleus incertus (NI) is a discrete region within the brainstem, situated in close proximity to the posterior aspect of the tegmentum. This region of the brain contains a diverse population of neurons that are involved in a range of functions, including stress response, arousal, learning, and modulation of the hippocampal theta rhythm. Additionally, orexin neuropeptides exhibit extensive distributions and overlapping actions within the NI. Nevertheless, the functions of orexin receptors within the NI remain poorly understood. The present study examined the effect of post-training and pre-probe intra-NI administration of SB-33486-A (OX1R antagonist) (12 µg/0.5 µl) and TCS-OX2-29 (OX2R antagonist) (10 µg/0.5 µl) on consolidation and retrieval in a Morris Water Maze (MWM) task. In Experiment 1, rats were trained in the Morris Water Maze (MWM) task and immediately after each training session received injections of dimethyl sulfoxide (DMSO) (control group), SB-334867-A, and TCS-OX2-29 into the nucleus incertus (NI). Experiment 2 was analogous to Experiment 1, with the exception that the rats received DMSO, SB-33486-A, and TCS-OX2-29 15 minutes prior to the probe test. In subsequent experiments, the probe and visible tests were conducted following the final training period, and the distance moved, escape latency, and velocity were recorded. In Experiment 3, rats that had undergone training in Experiments 1 and 2 were immediately subjected to trials for the assessment of visuomotor coordination on the visible platform. The results demonstrated that the spatial reference memory consolidation phase was markedly impaired by SB-334867-A or TCS-OX2-29 ($P < 0.05$), whereas the retrieval phase remained unaltered ($P > 0.05$). In light of these findings, it can be concluded that the orexinergic system in the NI plays a pivotal role in consolidation in rats through both OX1 and OX2 receptors.

Keywords: Orexin receptors; Nucleus incertus; Morris water maze; Memory

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1. Introduction

The nucleus incertus (NI) is situated in the midline of the prepontine region of the rat brain, in close proximity to the dorsal raphe (1). The majority of NI neurons contain GABA and a range of co-transmitters, including relaxin-3 (2). Some glutamatergic projections arising from this nucleus have been identified within the septo-hippocampal system (3). The presence of CRF1 receptors within the NI indicates that this area plays a role in stress-related responses (4, 5). The NI outputs project to the hippocampus, amygdala, the nucleus of the diagonal band, and several other regions, while the main inputs derive from the medial septum, habenula, raphe nuclei, and the contralateral NI (6). The diverse inputs and outputs have prompted researchers to conduct studies of the NI concerning stress, nutrition-related behavior, and arousal, as well as the effects of NI activity on the hippocampal theta rhythm (7-11). This perspective indicates that NI involvement in the generation of the hippocampal theta rhythm implies a role for the NI in the learning and memory processes within the hippocampus. The NI contains orexinergic fibers, and orexin 1 and 2 receptors (OX1R and OX2R, respectively) are expressed by NI neurons (12). The neurons responsible for the production of orexin-A and orexin-B peptides originate from a single precursor, prepro-orexin, and are concentrated in a limited region of the hypothalamus. The action of these peptides is mediated by OX1R and OX2R (13, 14). The OX1R has a 10-fold greater propensity for orexin-A than orexin-B (15). Orexin is a significant neurotransmitter implicated in a multitude of functions, including the sleep-wake cycle, feeding behavior, motivation and reward-related actions, alertness, memory, and learning (16, 17). In this regard, the involvement of orexins and their roles in learning and memory processes have been demonstrated. The inactivation of orexin receptors in the hippocampus, a region crucial for behaviors necessitating working memory, impairs memory recall by inhibiting the encoding, consolidation, and retrieval of spatial reference memories during the Morris water maze task. Furthermore, this investigation (18) has demonstrated that orexin deficiency impairs spatial working memory in mice. Nevertheless, the function of the orexinergic system in memory processes in NI remains uncertain. Accordingly, the objective of this study was to assess the impact of OXR1 and OXR2 inactivation in NI on spatial memory in rats.

2. Materials and Methods

2.1. Animals and Ethical Approval

Adult male Wistar rats (n=42; 250–350 g body weight) were obtained from the Laboratory Animal Institute of Mazandaran University of Medical Science. The rats were housed in groups of three per cage at a temperature of 23 ± 2°C under a standard 12-hour light/dark cycle, with ad libitum access to water and food. Behavioral training or testing was conducted between 7:00 a.m. and 2:00 p.m. All tests were performed in accordance with the Declaration of Helsinki and the internationally accepted ethical principles for the experimental use of animals. The study was approved by the Ethics Committee of Mazandaran University of Medical Sciences with Ethics Code IR.MAZUMS.REC.

2.2. Surgical Procedures and Microinjection of Drugs Into the NI

The rats were anesthetized via intraperitoneal injection of a ketamine-xylazine mixture (100 and 2.5 mg/kg, respectively). They were then placed into the stereotaxic frame, and a cannula was slowly inserted into NI (ML: 0, AP: -9.8, DV: 7-7.5) and fixed to the skull bone with dental cement. These coordinates were derived from the Paxinos and Watson rat brain atlas (19). The rats were permitted to recuperate for a period of one week prior to the commencement of the behavioral tests. Microinjections were conducted via the guide cannula (22 gauge) using a Hamilton syringe, which consisted of a polyethylene tube (10 cm length) fitted into an injection needle (27 gauge). Subsequently, 0.5 µl of dimethyl sulfoxide (DMSO), 12 µg of SB-334867-A in 0.5 µl, or 10 µg of TCS-OX2-29 in 0.5 µl was injected into NI. The infusion was conducted over a three-minute period, and the needle was left in place for one minute following the injection. DMSO has been demonstrated to have no significant impact on Morris water maze (MWM) learning and memory (15, 20, 21).

2.3.Morris Water Maze Apparatus

The apparatus is a black cylindrical pool with a diameter of 150 cm and a height of 50 cm, filled to a depth of 30 cm with water maintained at a temperature of 25 ± 2 °C. The pool was hypothetically divided into four equal quadrants by two principal axes, designated as south-east (SE), north-east (NE), south-west (SW), and north-west (NW). A Plexiglas platform, with a diameter of 11 cm, was positioned in the center of the SW quadrant (target quadrant) of the pool, at a distance of 15 cm from the edge and at a depth of 2 cm below the surface of the water. To assist with spatial orientation, a number of geometric cues were affixed to the adjacent walls of the pool. A camera was positioned above the central area of the pool to record the movements and behavior of the test rats. The room lighting was adjusted to approximately 50 lux to prevent additional reflection and reduce stress.

2.4. Adaptation

Prior to the commencement of the testing procedure, the rats were acclimated to the MWM environment to minimize stress levels. This was achieved by allowing the rats to swim the maze for 60 seconds without a platform.

2.5. Procedure (Hidden Platform Testing)

The training was conducted in two distinct consolidation and retrieval phases. The rats were trained over the course of three days, with two training blocks per day and four trials per block (Frey and Morris, 1997) (block interval: 15 minutes, trial interval: 2 minutes). In each trial, the rat was released into one of the quadrants and permitted to reach the location of the hidden platform within a 65-second time frame. In the event that the rat was unable to reach the

platform within the allotted time, it was guided at a slow pace to the platform. Once the platform was located, the rat was permitted to remain in that location for 15 seconds, during which time it could identify the position of the platform by viewing the visual cues. In both the consolidation and retrieval phases, the rats were divided into three groups: a control group (n=8), an SB334867-A group (n=8), and a TCS-OX2-29 group (n=8). Immediately following the conclusion of the training phase, which consisted of two blocks per day, each rat was administered a 0.5 µL infusion of the drug or a solution of dimethyl sulfoxide (DMSO) as a control. Following a three-day training and infusion period, a probe test was conducted 24 hours later. In the retrieval phase, rats were trained in a three-day protocol comprising two blocks per day, without any infusion. Fifteen minutes prior to the commencement of the probe test, the rats were administered 0.5 µL of SB334867-A, TCS-OX2-29, or DMSO (control group) via injection. Subsequently, the rats were subjected to a 65-second probe trial.

2.6. Behavioral Experiments

2.6.1. Probe Test

Twenty-four hours after the conclusion of all training sessions, the platform was removed. The rats were released from one of the maze quadrants, situated opposite the location of the platform, and were permitted to swim freely in the tank for a period of 65 seconds. This test is predicated on the assumption that the rat will recall the location of the platform and spend the majority of its time in the quadrant of the maze.

2.6.2.Visual Test (Visible Platform Testing)

The visible test was conducted 30 minutes following the probe test. The platform was situated at a height of one centimeter above the water level in the center of the quadrant in front of the target quadrant, and all visual cues were removed. The objective of this test was to ascertain whether sensory-motor factors and visual disturbances might exert an influence on the rat's capacity to reach the platform.

2.7.Experimental Design

2.7.1. Experiment 1 (Consolidation Phase of Spatial Memory)

The objective of this experiment was to assess the impact of OX1R and OX2R inactivation in the NI on the consolidation phase, and to evaluate the effect of SB334867-A or TCS-OX2-29 injection into the NI region on spatial memory through a probe trial. In order to minimize stress levels, rats were habituated for one week following surgery to the implantation of a guide cannula, which was used for the consolidation phase. Following the habituation period, the rats were randomly assigned to one of three groups: an SB group (n=8), a TCS group (n=8), and a control group (n=8). Each group was then trained for three consecutive days. Immediately following each training session, the rats were administered a 0.5 µL infusion of the antagonists or vehicle. The probe test was conducted 24 hours later, and the distance traversed and escape latency were recorded. The visible test was conducted 30 minutes after the probe test (Figure 1).

2.7.2. Experiment 2 (Retrieval Phase of Spatial Memory)

The objective of this test was to evaluate the influence of SB334867-A or TCS-OX2-29 injection into the NI region on the retrieval phase of spatial learning. One week following cannulation, rats underwent training. Following a three-day training period, the rats were randomly assigned to one of three experimental groups: control (n=8), SB334867-A (n=8), and TCS-OX2-29 (n=8). One microliter of the test substance (SB334867-A, TCS-OX2-29, or DMSO, serving as the control) was administered intraperitoneally 15 minutes prior to the probe test, which occurred 24 hours after the conclusion of the training period. The data pertaining to the escape latency and the distance traversed by the subjects during the course of the test were duly recorded. The visible test was conducted 30 minutes after the probe test (Figure 2).

2.7.3. Experiment 3: Visible Platform Test

Immediately after the probe test in experiments 1 and 2, rats were subjected to a visual platform task to investigate the possible interference of any visual disturbances on their motor function and motivation to reach the platform.

2.8. Confirmation of Correct Targeting of the NI Area

Following the behavioral tests, methylene blue was administered via the NI, and the rat was subsequently decapitated. The cannula was then carefully removed, and the brain was immersed in 10% formaldehyde for 7-10 days, after which the data were analyzed. Only the data from the rats with the cannula located in the NI were included in the analysis.

2.9. Data Analysis

The data were analyzed using GraphPad Prism 8 software. For the multiple comparison tests, one- and two-way ANOVA and Tukey's test were employed. The general behavior of the rats in the experimental groups was evaluated by measuring the average escape latency time and the distance traveled (mean ± standard error of the mean (SEM)). A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Experiment 1

3.1.1. Training Days

In this experiment, an intra-NI injection of DMSO (0.5 µl), SB-334867-A (12 µg /0.5 µl), or TCS-OX2-29 (10 µg /0.5 µl) was administered at the conclusion of each training day. The time required for rats to reach the platform on each of the three training days was recorded. The data were analyzed using a two-way repeated-measures ANOVA, which revealed no statistically significant interaction between the treatment groups and training days in terms of mean escape latency ($F(4, 28)= 2.239$; $P=0.0902$) (Figure 3A). Furthermore, no significant difference was observed across each training trial on day 1 ($F(14, 98) = 0.5016$; $P = 0.9271$) (Figure 3B) or day 2 ($F(14, 98) = 0.8481$; $P = 0.6161$) (Figure 3C). However, a significant interaction was observed between treatment groups and training trials on day 3 ($F(14, 98) = 2.134$; $P=0.0160$) (Figure 3D). In contrast, the Tukey multiple comparisons test revealed a notable difference in escape latency on day 2 between the TCS and DMSO groups in trials 1, 2, and 4 ($p=0.0014$, $p=0.0208$, and $p = 0.0127$, respectively).

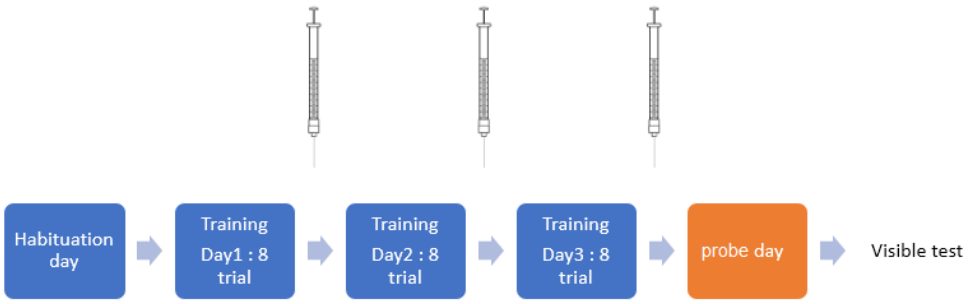

Figure 1. Overview of the consolidation phase

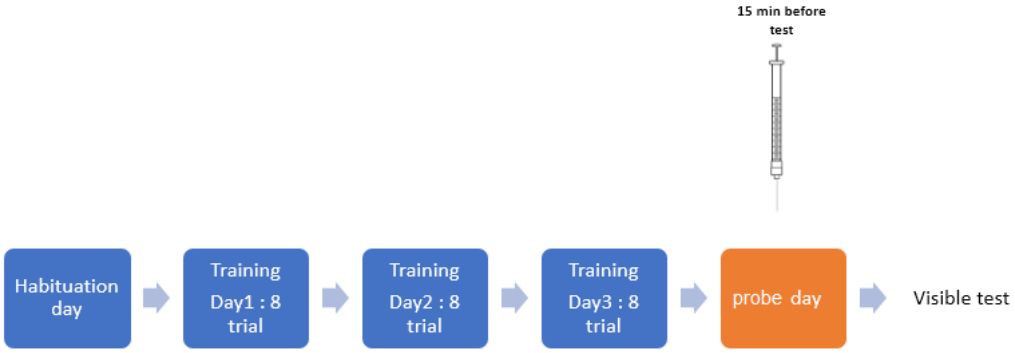

Figure 2. Overview of the retrieval phase

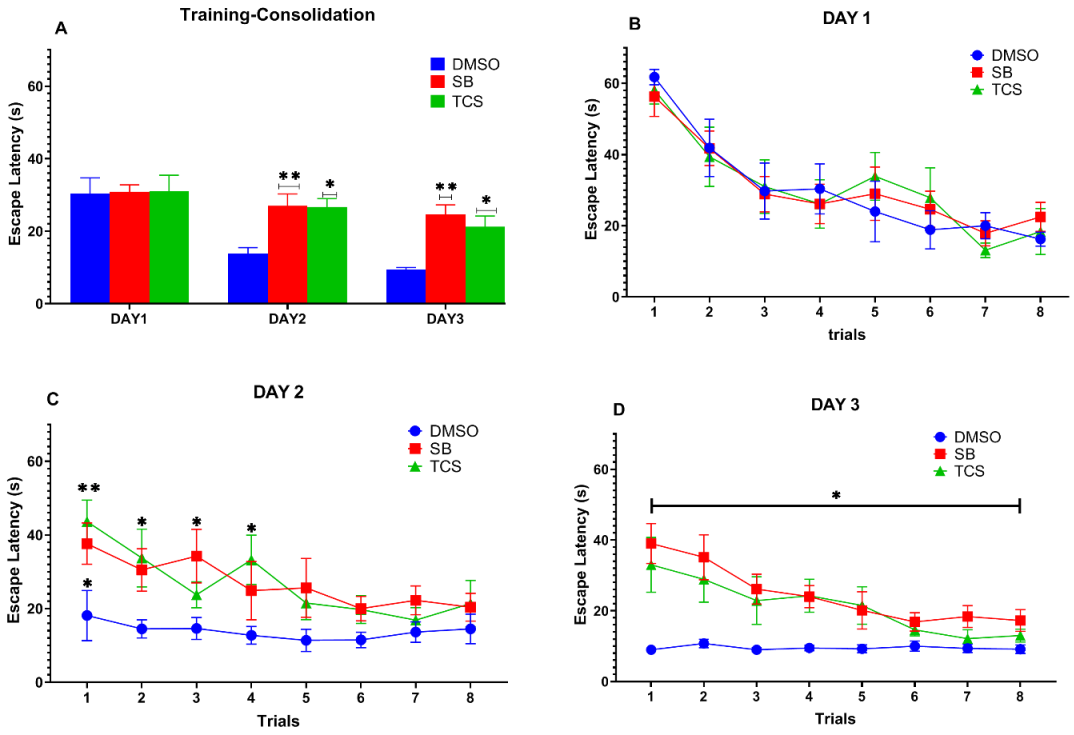

Figure 3. The effect of post-training intra-NI injection of DMSO (dimethyl sulfoxide), SB (SB-334867-A), and TCS (TCS, TCS-OX2-29) on the spatial learning consolidation phase: (A) average time to reach the platform within the three training days. Immediately after each training session, each rat received DMSO or SB, or TCS infusion on days 1, 2, and 3. (B-D) A comparison between groups of the time to reach the platform. Data are mean ± standard error of the mean (SEM) (*P < 0.05 and **P < 0.01). Data significance was determined by comparing the escape latencies with the DMSO group for eight rats in each group.

Furthermore, the results of this test demonstrated a notable discrepancy in escape latency between the SB and DMSO groups in trials 1 and 3 (P=0.0189 and P=0.0180, respectively) (Fig. 3C). A two-way repeated-measures ANOVA revealed a significant association between the distance traveled and the treated groups based on the training days (F (4, 28) = 3.173; P=0.0286) (Figure 4A). Furthermore, the repeated measures ANOVA revealed no significant interaction among the groups during each training trial on days 1 and 2 (Fig. 4B and Fig. 4C). The ANOVA yielded the following results: on day 1 (F (14, 98) = 0.2550; P=0.9969), and on day 2 (F (14, 98) = 0.7881; P=0.6796). However, a significant interaction was observed between treatment groups and training trials on day 3 (F (14, 98) = 2.708; P=0.0021) (Figure 4D).

3.1.2. Probe day

A probe test was conducted one day after the training days to assess the impact of SB-334867-A (12 µg/0.5 µl), TCS-OX2-29 (10 µg/0.5 µl), and DMSO (0.5 µl) on the consolidation phase of spatial memory in the MWM. The times required for rats in the antagonist groups to reach the target zone were significantly shorter than those of the rats in the DMSO group (F (6, 42) = 3.947; P=0.0032) (Fig. 5A). In contrast to the rats in the DMSO group, the rats in the antagonist treatment groups did not recognize the target quadrant. The analysis of the escape latency (F (2, 21)=6.042; P=0.0085) to the eliminated platform by Tukey's multiple comparisons revealed a significant difference between the SB (P=0.0300) or TCS (P = 0.0113) groups and the DMSO group (Figure 5B).

3.2. Experiment 2

3.2.1. Training Days

The rats were trained for a period of three days without receiving an injection. Subsequently, 15 minutes prior to the probe trial, the rats were administered injections of DMSO (0.5 µl), SB-334867-A (12 µg/0.5 µl), or TCS-OX2-29 (10 µg/0.5 µl) into the NI. A one-way ANOVA repeated measure (RM) was employed to analyze the data, which demonstrated a significant difference in the time taken to reach the platform on days 2 and 3 (F (23, 46) = 3.910; P < 0.0001) compared to day 1 (Fig. 6A). A one-way repeated measures ANOVA revealed a significant difference in the distance traveled by the rats in the different groups (F (23, 46) = 4.662; P < 0.0001). Further analysis with Tukey's multiple comparisons tests indicated that the distance traveled by the rats on the second and third days was less than that on the first day (P <0.0001), suggesting that the task was learned by the rats within the three training days.

3.2.2. Probe Day

In this experiment, the effects of antagonists injected into the NI just 15 minutes before the probe trial were evaluated during the retrieval phase of spatial memory in the MWM. The antagonists used were DMSO (0.5 µl), SB-334867-A (12 µg /0.5 µl), and TCS-OX2-29 (10 µg /0.5 µl). The time spent by the rats in the target quadrant was not significantly different between the antagonists' groups and the DMSO group (F (6, 42) = 0.3549; P = 0.9030). Accordingly, the rats in the DMSO, SB, and TCS groups demonstrated recognition of the target quadrant (Figure 7A). The analysis of the escape latency to the removed platform in the probe session by Tukey's multiple comparisons revealed no significant difference between the SB or TCS group and the DMSO group (F (2, 21) = 1.902; P=0.1740; Figure 7B).

3.3. Visible Platform Test

Following a 30-minute interval, rats from Experiment 1 were subjected to a visual platform task. The escape latency of the SB or TCS groups was not statistically different from that of the control (DMSO) group (F (2, 21) = 0.06381; P=0.9384) (Figure 8A). Additionally, the swimming speed of the SB or TCS groups was not significantly different from that of the control (DMSO) group (F (2, 21) = 0.06072; P=0.9413) (Figure 8B). In Experiment 2, the escape latency of the TCS and SB groups was not significantly different from that of the control (DMSO) group (F (2, 21) = 0.1733; P=0.8421) (Figure 9A). Moreover, the swimming speed of the SB and TCS groups was not significantly different from that of the control (DMSO) group (F (2, 21) = 0.6330; P = 0.5408) (Figure 9B).

4. Discussion

This is the inaugural study to demonstrate the impact of OXR1 and OXR2 deactivation in the NI region on spatial memory processing in rats. The NI comprises a heterogeneous population of neurons that express multiple neuropeptides and receptors (22). Nevertheless, the precise physiological and anatomical characteristics of NI neurons and their projections remain unclear. The results demonstrated that the post-training infusion of TCS-OX2-29 or SB-334867-A into the NI region markedly hindered the consolidation phase of spatial memory, as evidenced by the rats' performance during the probe test and on training days. This was manifested by an increased escape latency and distance traveled. This impairment was observed in the initial trials of days two and three, as well as on the probe day. These results align with those of previous studies that demonstrated the disruptive effects of NI manipulation on diverse learning and memory processes, including passive avoidance learning, working and reference spatial memory, and the induction of LTP in the hippocampus associated with learning and memory (21, 23, 24). The preceding studies indicate that NI plays a crucial role in regulating the septohippocampal system and theta rhythm in the hippocampus (22). It can thus be hypothesized that the inactivation of orexin receptors may affect this pathway through a similar mechanism. Furthermore, a previous study demonstrated that orexin receptor blockade in the medial septum resulted in comparable disruptive effects on the consolidation phase of reference memory (unpublished data). Additionally, evidence indicates that distinct orexin neuron populations converge on NI neurons, and orexin-A-containing axons form synaptic connections with relaxin-3-positive neurons in the NI region (12).

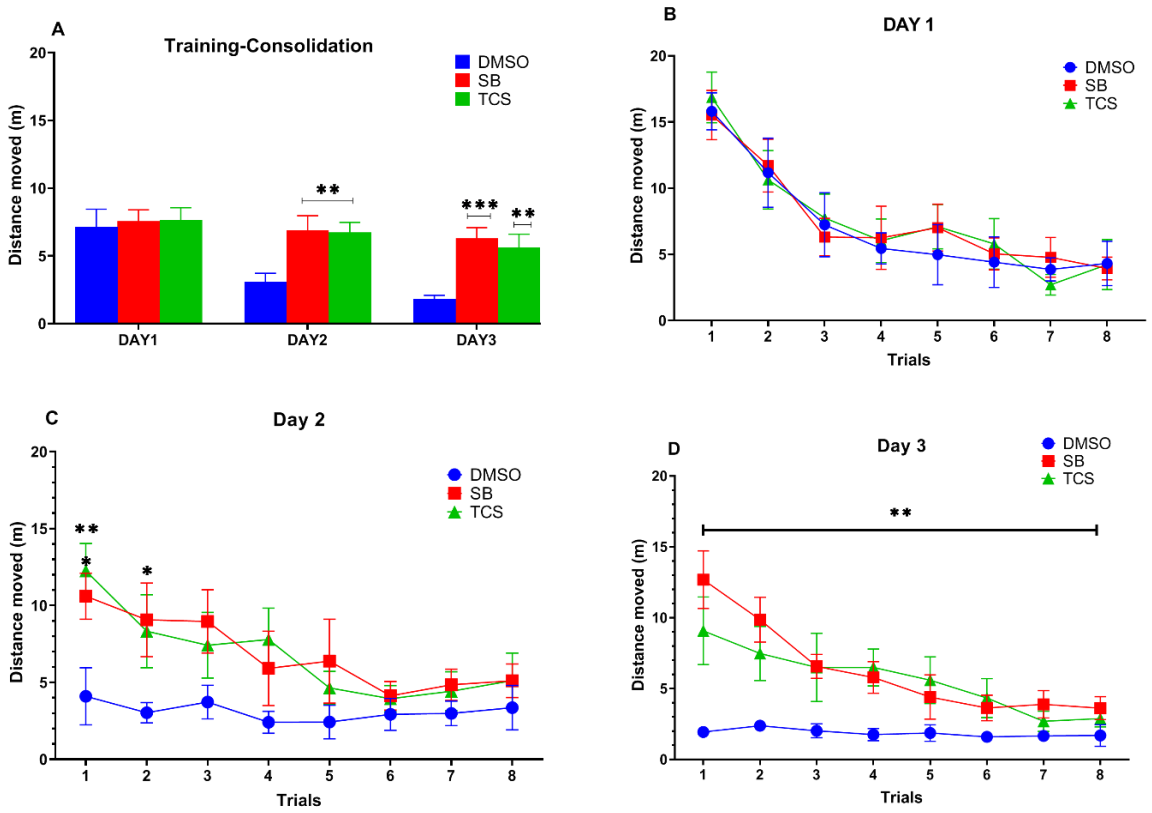

Figure 4. The effects of post-training intra-NI injection of DMSO, SB, or TCS on the spatial learning consolidation phase. (A) distance traveled by the rats during the 3 training days. Immediately after each training session, each rat received DMSO or SB, or TCS infusion on days 1, 2, and 3. (B-D) A comparison between groups of the distance traveled by the rats to reach the platform. Data are mean ± SEM (*P < 0.05, **P < 0.01 and ***P < 0.001). Data significance was determined by comparing the distance traveled with the DMSO group for 8 rats in each group.

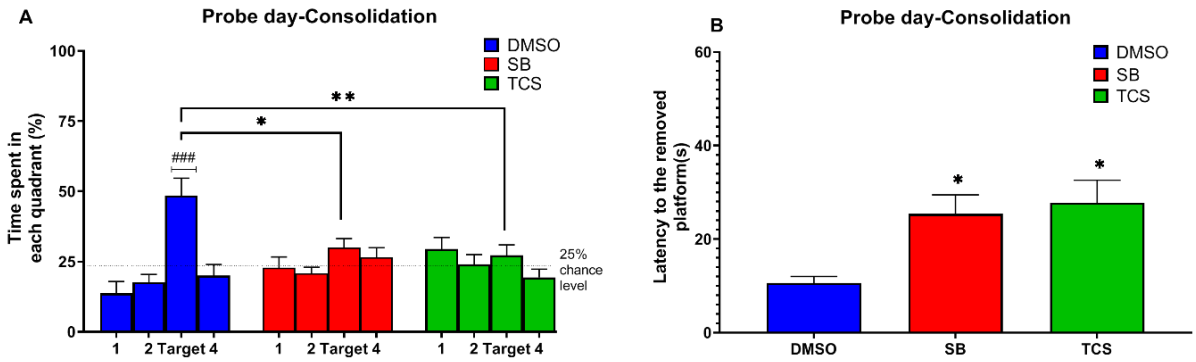

Figure 5. The effect of post-training intra-NI injection of DMSO, SB, or TCS post-training on the spatial learning consolidation phase. (A) The percentage of time spent in the target area by the rats during the probe test. (B) latency to the eliminated platform. Tukey's multiple comparisons analysis revealed a significant difference between the antagonist groups and the DMSO group during the consolidation phase (A). In A, columns represent as mean ± SEM. Escape latency (*P < 0.05 and **P < 0.01) is statistically significant for the DMSO group. ###P < 0.001, the difference between the target and the other three quadrants compared to different quadrants in the DMSO group.

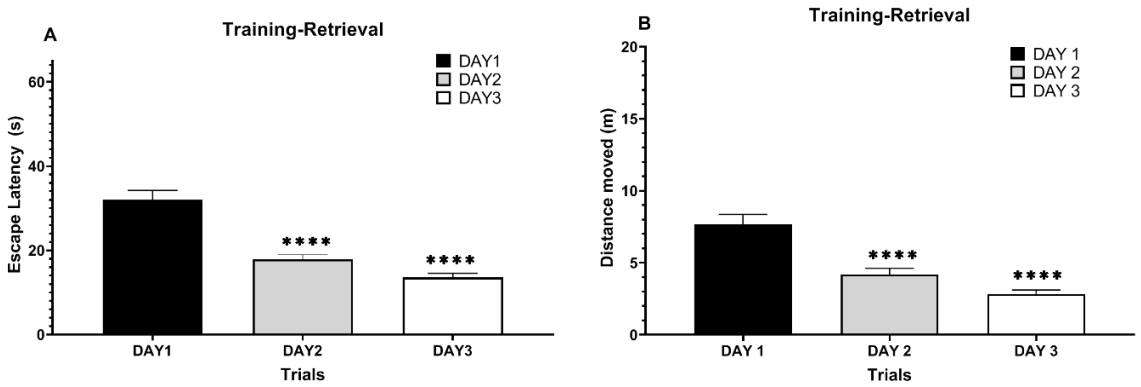

Figure 6. (A) The escape latency and (B) distance traveled by experiment 2 rats trained for 3 days without injection. Data analysis by Tukey's multiple comparisons and one-way repeated measures ANOVA tests demonstrated significant changes in escape latency and distance moved on days 2 and 3 compared to day 1. Data are present as mean ± SEM. ****P < 0.001, significant in comparison to day 1.

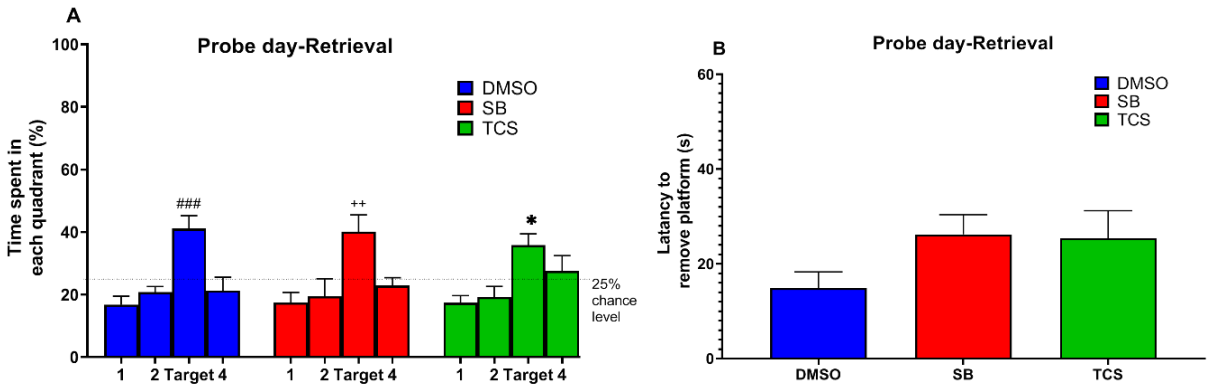

Figure 7. The effect of pre-probe test intra-NI injection of DMSO, SB, or TCS on the spatial learning retrieval phase: (A) The percentage of time spent in the target area by the rats during the probe test. (B) latency to the eliminated platform. Tukey's multiple comparisons analysis did not reveal a significant difference between the antagonist and DMSO groups in the retrieval phase (A). In A, each column is the mean ± SEM. ###P < 0.001, significance level compared with the different quadrants in the DMSO group. ++P < 0.01, significance level compared to different quadrants in the SB group, and *P < 0.05 compared to different quadrants in the TCS group.

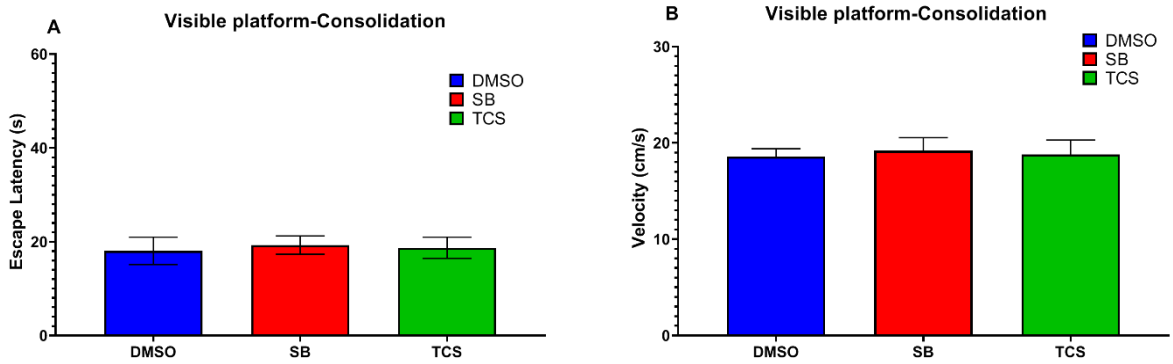

Figure 8. The visual platform task (A) escape latency and (B) swimming speed; for Experiment 1 rats. Intra-NI injection of DMSO and SB, and TCS did not produce any significant change in the swimming speed or escape latency to that displayed by the DMSO group. Data are presented as mean ± SEM.

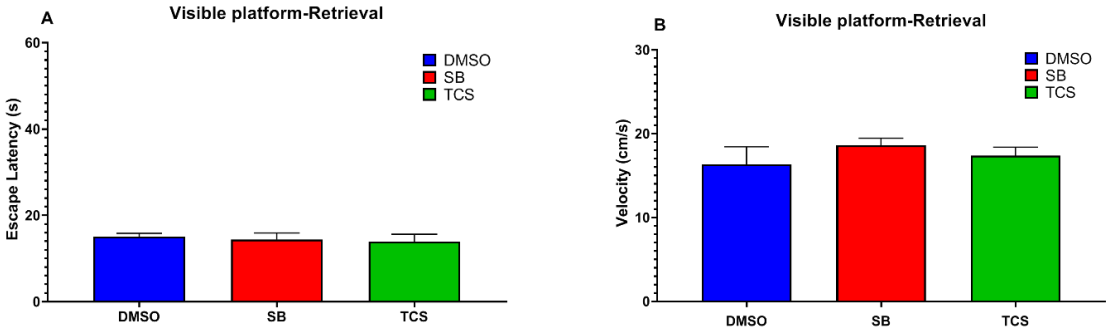

Figure 9. (A) The escape latency and (B) swimming speed during the visual platform task for Experiment 2 rats. Intra-NI injection of SB and TCS did not produce any further change in swimming speed or escape latency than the DMSO vehicle. Data are expressed as mean±SEM.

Previously, it was demonstrated that relaxin-3 is capable of modulating arousal, the stress response, feeding, and memory, and may be involved in the generation of the hippocampal theta rhythm (25). In light of these findings, it can be proposed that orexin in NI may contribute to memory processes through its overlap with relaxin-3. Additionally, the findings of Experiment 2 (retrieval phase) in this study indicate that post-training infusion of SB-334867-A or TCS-OX2-29 into the NI region did not exhibit notable differences between treatments and did not influence the retrieval phase of spatial reference memory. This is in contrast to the impact of orexin receptors inactivation in the medial septum (unpublished data). Moreover, the current results align with those of previous studies, indicating that orexin receptor inactivation in the NI does not impair the retrieval phase of the MWM task after 24 hours. In contrast, a previous study reported that NI inactivation delayed learning and impaired retrieval in the MWM, which is inconsistent with the results of the present study (23). In this regard, the complete inactivation of this region by lidocaine (a sodium channel blocker) affected all outputs from the NI, accompanied by significant cognitive deficits in spatial learning. Nevertheless, it has been demonstrated that the inactivation of the NI by lidocaine following the application of high-frequency stimulation did not influence the maintenance of both pEPSP and PS-LTP. Moreover, the results of the visible platform test demonstrated that orexin receptor blockade within the NI did not influence escape latency and velocity in a non-spatial visual discrimination task, nor did it disrupt the rats' visual or motor activities. This finding corroborates the presence of cognitive and learning impairments observed in the aforementioned experiments. In conclusion, the results of this study indicate that the inactivation of NI OX1R and OX2R affects the consolidation phase of spatial memory, as evidenced by a reduction in the time spent by animals in the target quadrant in MWM and an increase in escape latency. Nevertheless, evidence for potential cellular and molecular mechanisms that may contribute to a reduction in the time spent by animals in the target quadrant is limited. In conclusion, our findings demonstrated for the first time that the inactivation of NIOX1R and NIOX2R impaired the consolidation phase of spatial memory in the MWM task, but had no effect on the retrieval phase.

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Authors' Contribution
Study concept and design: E. A. and V.B.
Acquisition of data: F. E.
Analysis and interpretation of data: F. E.
Drafting of the manuscript: F. E., E.A. and M.Z.
Critical revision of the manuscript for important intellectual content: F. E., E.A. and M.Z.

Ethics
The experimental procedure was approved by the Mazandaran University of Medical Sciences in Sari, Iran.

Conflict of Interest
The authors certify that they have no conflicts of interest.

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

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