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# Orexin-A-induced $ERK_{1/2}$ activation reverses impaired spatial learning and memory in pentylenetetrazol-kindled rats via OX1R-mediated hippocampal neurogenesis

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ABSTRACT

Epilepsy is characterized by the occurrence of repetitive seizures and can greatly affect a patient's cognition, particularly in terms of learning and memory. Orexin-A is an excitatory neuropeptide produced by the lateral hypothalamus that has been shown to be involved in learning and memory. A reduction in the levels of orexin-A after seizures may underlie the learning and memory impairments induced by epilepsy. Thus, we used pentylenetetrazol (PTZ)-kindled rats to investigate the effects of orexin-A on learning and memory and the involvement of neurogenesis in the dentate gyrus in OX1R-mediated $ERK_{1/2}$ activation. A Morris water maze test revealed reduced escape latencies, prolonged times in the target quadrant and an increased number of platform crossings in PTZ-kindled rats exposed to orexin-A. These ameliorating effects of orexin-A on spatial learning and memory were attenuated by the intracerebroventricular injection of the OX1R antagonist SB334867 or the $ERK_{1/2}$ inhibitor U0126. Further studies using bromodeoxyuridine (BrdU) revealed that orexin-A increased the number of BrdU-positive cells, doublecortin (DCX)/BrdU levels and the number of NeuN/BrdU double-positive nuclei in the dentate gyrus of PTZ-kindled rats. However, these effects were inhibited by treatment with SB334867 or U0126. Taken together, these data suggest that orexin-A attenuated the impairment of spatial learning and memory in PTZ-kindled rats and that this attenuation involved neurogenesis in the dentate gyrus via OX1R-mediated $ERK_{1/2}$ activation.

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## 1. Introduction

Epilepsy is a common neurological disease that is characterized by repeated spontaneous seizures. In addition to recurrent seizures, patients with epilepsy frequently exhibit cognitive impairments, particularly in learning and memory. Clinical studies have demonstrated that learning and memory in epileptic patients are affected by several factors, including the neuroanatomical localization, frequency and duration of the seizures; the patient's age; and the anti-epileptic drugs that have been administered [8,15]. In addition, these impairments in learning and memory depend on the type of epilepsy and its etiopathology [8]. Pentylenetetrazol (PTZ)-induced seizures have been shown to result in hippocampal atrophy and neuronal loss in limbic areas, leading to deficits in learning and memory [25]. Further studies have demonstrated that the attenuation of spatial learning and memory observed in PTZ-kindled rats involves increased concentrations of intracellular calcium, decreased levels of total CaMKIIα and phosphorylated CaMKIIα (P-CaMKIIα) and decreased phosphorylation of cAMP response element-binding protein (P-CREB) in the hippocampus [40]. However, the underlying mechanisms of these changes remain unclear. Recently, studies of the orexin system in the hippocampus have shown that this system is involved in learning and memory [17]. Moreover, orexin-A concentrations are reduced following epileptic seizures, which suggests that lower orexin-A levels in the brain may be associated with the attenuation of learning and memory caused by epilepsy [27].

Orexin peptides (orexin-A and orexin-B) are produced by the lateral hypothalamus and function in the regulation of feeding, energy homeostasis, neuroendocrine activities and the sleep-wake cycle by activating the orexin-1 (OX1R) and orexin-2 (OX2R) receptors [14,29,36,45]. The projection of orexinergic neurons to the hippocampus, where OX1R and OX2R are widely expressed, has been implicated in learning and memory [1,17,38]. The nasal administration of orexin-A alleviates cognitive deficits caused by sleep deprivation and restores social memory in orexin/ataxin-3-transgenic mice [44], suggesting an important role for orexin-A

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in learning and memory. The regulation of cholinergic activity in the hippocampus by the orexin system further suggests this pathway's involvement in cognition. In vitro, the orexins have been shown to regulate cell proliferation and differentiation by activating extracellular signal-regulated kinase$_{1/2}$ (ERK$_{1/2}$) via OX1R and OX2R [20,37]. Studies of behavior and hippocampal synaptic plasticity indicate that ERK$_{1/2}$ activation enhances the induction of long-term potentiation (LTP), which contributes to the formation of memories [32]. However, whether orexin-A-induced ERK$_{1/2}$ activation via orexin receptors improves the learning and memory deficits induced by epilepsy remains unclear.

In the present study, we investigated the hypothesis that the improvement in learning and memory in epilepsy mediated by orexin-A involves OX1R-mediated ERK$_{1/2}$ activation and hippocampal neurogenesis. We established a model of PTZ-kindled rats and evaluated the spatial learning and memory abilities of these rats with the Morris water maze (MWM) test. Additionally, bromodeoxyuridine (BrdU) and immunofluorescence analyses were used to investigate the ability of orexin-A to ameliorate spatial learning and memory deficits and enhance hippocampal neurogenesis in epileptic rats. The OX1R antagonist SB334867 and the ERK$_{1/2}$ inhibitor U0126 were used to evaluate the mechanism by which orexin-A-induced ERK$_{1/2}$ activation via OX1R affects learning and memory in association with neurogenesis in the dentate gyrus of PTZ-kindled rats.

## 2. Materials and methods

### 2.1. Animals

Adult male Wistar rats (200–250 g) were purchased from Shandong Medical University Experimental Animal Center (Jinan, China) and housed at the Qianfoshan Hospital Experimental Animal Center (Jinan, China). The animals were maintained on a 12-h light/dark cycle at a constant temperature (23 ± 2 °C) with ad libitum access to water and food. Animal care, surgery and handling procedures were approved by the Qianfoshan Hospital Animal Care Committee of Shandong University [protocol no. SYXK (Lu) 20120003] and were performed according to the principles of Laboratory Animal Care.

The animals were randomly divided into the following seven groups ($n$ = 12 per group): (1) control group with an injection of normal saline (NS) (NS + NS), (2) epileptic control group (PTZ + NS), (3) orexin-A group (PTZ + OXA), (4) SB334867 group (PTZ + SB), (5) U0126 group (PTZ + U0126), (6) U0126 + orexin-A group (PTZ + OXA + U0126) and (7) SB334867 + orexin-A group (PTZ + OXA + SB). According to the methods previously described by Smialowski and Diehl R.G. [10,33], epilepsy was induced in rats via repeated intraperitoneal (i.p.) injections of a sub-convulsive dose (35 mg/kg) of PTZ at 9 am every 24 h for 30 days until the kindling criterion was achieved, after which the rats' responses were measured according to the following five stages: no effect-0; facial movements, sniffing, head nodding, running-1; clonic forelimb convulsions-2; clonic convulsions with rearing-3; generalized clonic convulsions with rearing and episodes of falling down-4; seizures with episodes of falling down and periods of tonus-5. Rats exhibiting seizure stages 4–5 for three consecutive days were regarded as fully kindled, and rats that were not fully kindled or that died during the kindling procedures were excluded from the experiments. The control group received an equivalent amount of saline.

To evaluate the effect of epilepsy on orexin-A, two additional groups ($n$ = 12 per group) were established: (1) a non-PTZ group and (2) a PTZ group.

### 2.2. Drugs

Orexin-A and the selective OX1R antagonist SB334867 were obtained from TOCRIS, Germany. The ERK$_{1/2}$ inhibitor U0126 was purchased from Promega (St. Louis, MO, USA). Orexin-A was dissolved in sterile saline solution (14 μg/8 μl), and SB334867 (6 μg/8 μl) and U0126 (3.5 μg/8 μl) were dissolved in dimethyl sulfoxide (DMSO) solvent (final concentration of DMSO 0.2%, v/v). PTZ, BrdU, DMSO and the sheep anti-BrdU antibody were purchased from Sigma (St. Louis, MO, USA). The mouse anti-NeuN antibody was purchased from Millipore (Billerica, MA, USA). The mouse anti-BrdU and goat anti-DCX antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rhodamine-conjugated rabbit anti-sheep secondary antibody was obtained from Jackson Immunoresearch (West Grove, PA, USA). The rhodamine-conjugated rabbit anti-goat and FITC-conjugated goat anti-mouse secondary antibodies were obtained from Boster (Wuhan, China).

### 2.3. Surgical and microinjection procedures

After being PTZ-kindled successfully, the rats were anesthetized (10% chloral hydrate [300 mg/kg]), fixed on a stereotaxic apparatus (Narishige, Tokyo, Japan) and then implanted with a stainless steel guide cannula into the right lateral ventricle (0.8 mm posterior to the bregma, 1.5 mm lateral from the midline and 2.5 mm ventral to the cortical surface). After surgery, the rats were housed separately, and experiments were performed after seven days. The dose of orexin-A was selected on the basis of our previous study of the improvement of learning and memory in PTZ-kindled rats (unpublished). The PTZ-kindled rats were treated with intracerebroventricular (i.c.v.) injections of 8 μl saline, 8 μl orexin-A, 8 μl SB334867 or 8 μl U0126 at a rate of 2 μl/min every 24 h with a microinjection pump followed by the Morris water maze test. After each injection, the needles were left in place for 5 min to allow for drug diffusion.

### 2.4. Morris water maze

A black circular tank (150 cm in diameter and 50 cm high) was filled with water (25–27 °C) to a depth of approximately 22 cm. A black platform (10 cm in diameter and 20 cm high) was placed at the bottom of the tank in the center of one of the four equal-area quadrants (the goal quadrant). The experimenter and extra maze cues, including the location of the doors, desks and chairs, were maintained in the same positions throughout the experiments, and the room was kept quiet. There were two components to the MWM: the place navigation test (PNT) and the spatial probe test (SPT).

#### 2.4.1. Place navigation test

For the training trial, the rat was randomly placed in one of the non-target quadrants with its head facing the wall of the maze. The rat was then trained to find the platform within 120 s and recorded using an overhead video camera linked to a computer for image analysis. The swimming paths of the rats, escape latencies (the time required to reach the platform) and average swimming speed were measured as dependent variables. If the rat failed to find the platform within 120 s, it was guided to the platform by the experimenter for a 30-s rest and the escape latency was recorded as 120 s. Each rat was placed in the water of each quadrant twice a day.

#### 2.4.2. Spatial probe test

This test measured the rats' spatial reference memory. After five days of training, the platform was removed from the maze. The rats were first placed in the quadrant opposite the goal quadrant and were allowed to swim freely for 120 s. The total time spent in the

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