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Activation of Orexin-A Receptors in the Periaqueductal Gray Increases Pain Tolerance in Healthy and Diabetic Male Rats

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ABSTRACT

Introduction: Diabetic neuropathic pain is a common complication of diabetes. While previous research has shown that direct administration of the neuropeptide orexin-A into the brain can elicit analgesic effects, the specific central nervous system regions and mechanisms mediating these pain-relieving actions remain incompletely characterized.

Methods: The current study used male Wistar rats to investigate the antinociceptive effects of administering orexin-A directly into the ventrolateral periaqueductal gray (vlPAG) region of the midbrain, assessed via the tail-flick test performed 5 minutes post-injection. The experiments compared the effects of intra-vlPAG orexin-A administration in both healthy control and diabetic animal models.

Results: In the control groups, the analgesic effects of intra-vlPAG orexin-A were found to be sustained over the 1-hour observation period. Importantly, orexin-A elicited a rapid and potent analgesic response in the diabetic animal groups as well.

Conclusion: Collectively, these findings suggest a key functional role for the vlPAG orexinergic system in modulating pain tolerance, with implications for the potential therapeutic targeting of this system in the management of debilitating neuropathic pain conditions like diabetic peripheral neuropathy.

Keywords: Pain, Tail-flick test, Orexin A receptor, Ventrolateral periaqueductal gray, Diabetic neuropathy**.**

INTRODUCTION

Peripheral neuropathy, characterized by heightened sensitivity to painful stimuli, is one of the most common complications affecting up to 60% of individuals with diabetes (1). This neuropathic pain manifests as a chronic or persistent condition, marked by alterations in pain perception, exaggerated responses to noxious stimuli (hyperalgesia), and abnormal sensitivity to previously non-painful sensations (allodynia) (2). Current first-line pharmacological interventions for diabetic neuropathic pain include tricyclic antidepressants, calcium channel modulators like gabapentin and pregabalin, topical lidocaine, opioid analgesics, and tramadol. However, the

efficacy of these standard treatment regimens remains limited, underscoring the need for the discovery and development of novel therapeutic candidates (3).

Orexin (also known as hypocretin) originates from a small population of neurons located in the lateral hypothalamic area. These orexin-producing neurons project broadly throughout the brain and even into the spinal cord, enabling them to contribute to diverse physiological functions beyond their well-characterized role in regulating wakefulness (4-7). Importantly, the orexin peptides, orexin A and orexin B, have demonstrated the ability to reduce nociceptive responses in animal models of thermal, inflammatory, and visceral pain (8, 9). This analgesic effect of orexin may be mediated in part through its influences on key pain-processing regions such as the locus coeruleus, a major source of ascending and descending noradrenergic projections (10), as well as the periaqueductal gray, which is heavily innervated by orexin neurons and expresses the orexin 1 receptor (11).

The current study aims to investigate the potential of orexin A to attenuate neuropathic pain in the context of diabetes. Using a rodent model of streptozotocin-induced diabetes, we sought to elucidate the effects of direct administration of orexin A on pain sensitivity in this disease state characterized by peripheral nerve dysfunction.

MATERIALS AND METHODS

Experimental subjects and ethical standards

Twenty-four male Wistar rats (weighting 250-300 g, Pasteur Institute of Iran, Tehran) were used. The animals were group-housed under controlled environmental conditions, maintained on a 12-hour light-dark cycle (lights on from 07:00 to 19:00 hours) with ambient temperature held between 22-25°C. Throughout the experimental period, the rats were provided ad libitum access to a standard rodent chow diet and sterile drinking water. All experiments were conducted in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH Publications No. 85:23, updated 1985), ensuring the humane treatment and ethical use of the animal subjects.

Surgical technique and recovery period

The surgical procedure began by inducing deep anesthesia in the rats through an intraperitoneal injection of sodium pentobarbital (10 mg/kg). To minimize both pain and bleeding, a local anesthetic solution containing a 0.2 ml combination of lidocaine and epinephrine (Persocaine E) was administered around the surgical area. The animals were then positioned within a stereotaxic apparatus (Stoelting, USA) to ensure precise surgical placement.

A linear incision was made along the midline of the scalp, exposing the surface of the skull. The bregma and lambda cranial landmarks were identified and carefully cleaned. The stainless steel guide cannula was implanted targeting the midbrain ventrolateral periaqueductal gray (vlPAG) region, with the following stereotaxic coordinates relative to bregma: antero-posterior = -7.8 mm, medio-lateral = +0.8 mm, and dorso-ventral = 6.4 mm, as per the rat brain atlas (12). The guide cannula was securely anchored using two stainless steel screws, and the incision was closed with dental cement.

Following the surgical procedure, the animals were allowed a one-week recovery period before the commencement of experiments.

Diabetes induction

Diabetes was induced in the experimental group through a single intraperitoneal injection of freshly prepared streptozotocin (STZ) at a dose of 60 mg/kg. Three days after STZ administration, a blood sample was obtained via tail prick, and the blood glucose levels were measured using a digital glucometer (AVAN AGM01®, Iran). Rats with blood glucose concentrations exceeding 250 mg/dL were classified as diabetic. The day on which hyperglycemia was confirmed was designated as the study's starting point. The control group received an equivalent volume of the citrate buffer vehicle. Throughout the study, the body weights and blood glucose levels of the rats were measured at both the onset and the end of the experimental period.

Drugs and mode of application

Two key pharmacological agents were utilized in this investigation:

Streptozotocin (STZ): STZ was acquired from Sigma–Aldrich Co. (USA). To prepare the diabetogenic agent, STZ was dissolved in 0.1 M sodium citrate buffer, with the pH carefully adjusted to 4.5. This STZ solution was administered to the experimental group via a single intraperitoneal injection at a dose of 60 mg/kg.

Orexin A: Orexin A was also obtained from Sigma–Aldrich Co. (USA). Orexin A was prepared by dissolving it in a normal saline solution (0.9 % NaCl).

For the intracerebral microinjections, a consistent volume of 0.5 µl was maintained across all experimental groups, irrespective of whether the injection contained the drug (Orexin-A: at dose of 10 nmol/0.5 µl) or the vehicle (normal saline). The microinjections were performed using a 1-µl Hamilton syringe connected to a stainless steel injector (30 gauge, 12 mm needle, 1 mm longer than the implanted guide cannulas) via a polyethylene tube (PE-20). The injections were precisely delivered over a duration of 50 seconds, and the injection cannulas were left in place for an additional 60 seconds to ensure optimal delivery of the administered substances into the target brain region, the vlPAG.

Pain assay (tail-flick test)

The nociceptive responses of the animals were assessed using the tail-flick test. The tail-flick test was performed using a dedicated apparatus (Poya armaghan Apparatus, Iran). During the test, the animal's tail was placed into the groove of the apparatus, and a thermal stimulus beam was directed onto the tail at two distinct locations: 3 cm and 5 cm from the tip of the tail. The latency between the application of the thermal stimulus and the withdrawal of the tail, defined as the tail-flick latency (TFL), was meticulously recorded. TFL measurements were obtained at multiple time points during the 60-minute experimental period: at 5, 15, 30, 45, and 60 minutes from the start of the experiment. To prevent potential tissue damage, a cut-off time of 10 seconds was imposed for the tail-flick response.

Experimental design

The animals were randomly allocated to control and diabetic groups. Each experimental groups were divided into two subgroups: a vehicle-treated group and an orexin-A-treated group. In each subgroup there were 6 animals. This experiment investigated the effects of intra-vlPAG orexin A on the duration of pain tolerance. The tail-flick test was performed after the animals were thoroughly fixed on the restraining device. Five min before the tail-flick test, animals were microinjected with orexin A or normal saline.

Histology

Following the completion of the tail-flick test, the animals were humanely euthanized. This was accomplished with deep anesthesia through the intraperitoneal administration of a mixture of Ketamine and Xylazine. After euthanasia, the brain of each animal was carefully removed and immersed in a 10% formalin solution. The brain samples were stored in the formalin solution for at least four days to ensure proper fixation. The fixed brain tissues were coronally sectioned into thin slices, each measuring 50 micrometers thick. These brain sections were subsequently analyzed using the Paxinos and Watson rat brain atlas as a reference guide. Only the data from animals with correctly positioned microinjection cannulae within the vlPAG region were included in the final statistical analysis.

Statistical analysis

All data are reported as the mean ± standard error of the mean (mean ± S.E.M.). Differences between experimental groups were assessed using a two-way analysis of variance (ANOVA), followed by Tukey's post hoc test for pairwise comparisons. Statistical significance was set at $p < 0.05$.

RESULTS

Orexin A microinjection into the vlPAG increases pain tolerance in healthy rats

The statistical analysis revealed a significant difference in tail-flick latency between the

experimental groups in healthy rats. At the 5-minute time point after the start of the test, the control group + orexin A exhibited significantly longer tail-flick latency compared to the control group + vehicle ($p < 0.01$). The significant difference in tail-flick latency between the orexin A and vehicle groups also persisted at the 15-minute ($p < 0.001$), 30-minute ($p < 0.001$), 45-minute ($p < 0.001$), and 60-minute ($p < 0.001$) time points following the start of the test (Fig. 1).

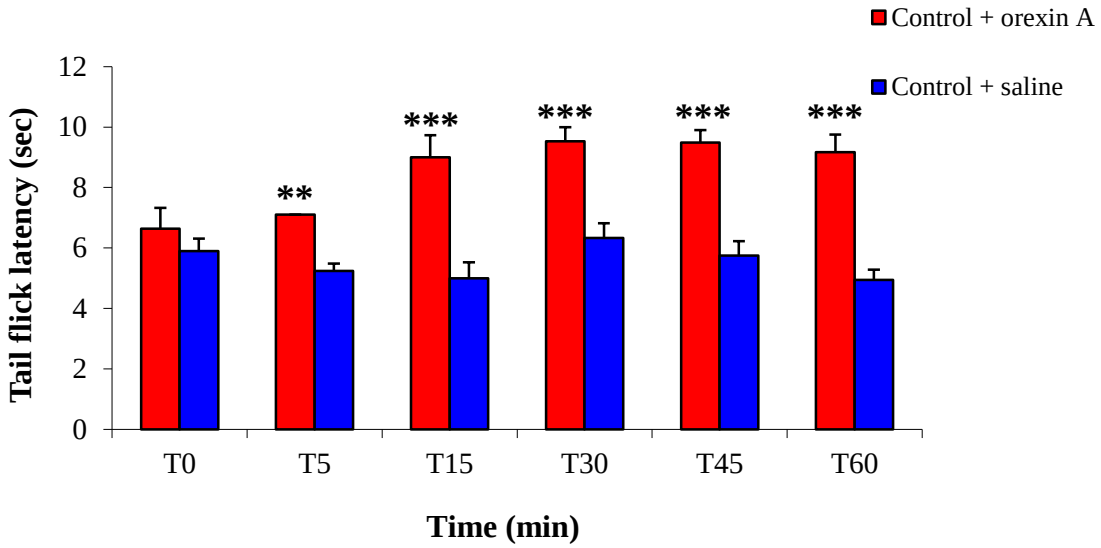

Fig. 1. The bar graph illustrates the impact of microinjection of orexin A into the ventrolateral periaqueductal gray (vlPAG) region in healthy animals (n=6). Data represent mean ± SEM. **p < 0.01, ***p < 0.001 compared with the control + saline group.

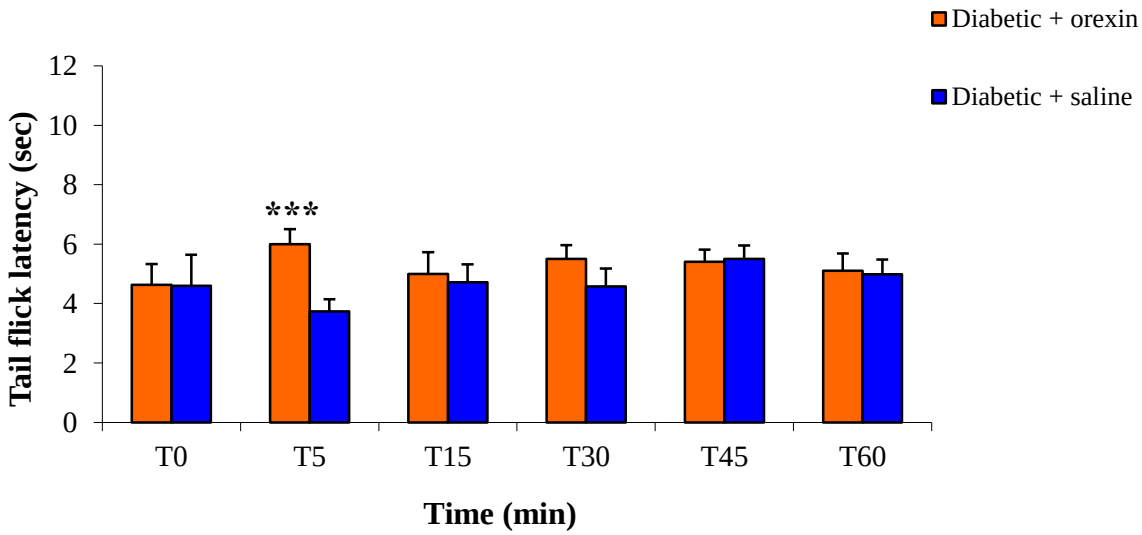

Fig. 2. The bar graph depicts the effect of microinjecting the neuropeptide orexin-A directly into the ventrolateral periaqueductal gray (vlPAG) region in the diabetic animal groups (n=6). Data represent mean ± SEM. ***p < 0.001 compared with the diabetic + saline group.

These findings demonstrate that the analgesic effects of intra-vlPAG orexin A administration were not transient, but rather maintained over the course of the 1-hour observation period.

Orexin A microinjection into the vlPAG increases pain tolerance temporarily in neuropathic rats

The analysis also revealed that administration of orexin-A significantly increased tail-flick latencies in the diabetic group compared to the diabetic control group receiving the vehicle treatment at the 5-minute time point ($p < 0.001$). This indicates that orexin A had a rapid analgesic effect in the diabetic animals. However, beyond the 5-minute time point, no significant differences were observed in tail-flick latency between the diabetic group that received orexin A and the diabetic control group (Fig. 2).

DISCUSSION

The site-specific administration of the neuropeptide orexin-A into the nuclei of the descending antinociceptive pathway offers a valuable experimental approach to investigate the role of this neurotransmitter system in the top-down modulation of pain processing. Orexin neurons are found exclusively within the dorsal and lateral regions of the hypothalamus (13, 14), areas of the brain that are well-established to play a key part in the modulation of nociceptive input through their extensive anatomical and functional connections with the PAG matter (15). Additionally, orexin-containing projections emanate widely throughout other brain regions that comprise the descending pain modulatory network, such as the PAG (16, 17).

In the present study, we clearly demonstrated that the microinjection of orexin-A directly into the PAG elicited robust anti-nociceptive effects, as evidenced by reduced nociceptive behaviors in the tail-flick test, in both healthy control animals and rodent models of diabetic neuropathy. Our findings align with an expanding body of research demonstrating that the orexin neuropeptides exert antinociceptive effects within the brain and spinal cord across diverse pain modalities. This includes attenuation of mechanical, chemical, and thermal nociception, as well as suppression of nociceptin-induced behavioral responses (18-20). For instance, Esmaili and colleagues have reported that the blockade of Orx1R specifically within the vlPAG is associated with a decreased latency in the tail-flick test following carbachol-induced antinociception (21). Zarmehri et al. have shown that the direct administration of orexin-A into the PAG exerts robust antinociceptive effects during both the interphase and late phase of the formalin test, an effect mediated through the Orx1R-dependent signaling cascade (22). Additionally, Niknia et al. have reported that orexin-A exerts anti-hyperalgesic and neuroprotective effects in a rodent model of diabetic peripheral neuropathy (23).

Several studies have established that the generation of free radicals and oxidative stress play critical roles in the development of neuronal apoptosis and neuropathy associated with diabetes (24). It has been demonstrated that high glucose concentrations can induce oxidative stress through the auto-oxidation of glucose, leading to the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). These mediators can then cause protein nitrosylation, lipid peroxidation, DNA damage, and apoptosis, with direct toxic effects on nerve tissue (25). Interestingly, previous research has shown that orexin-A possesses potent antioxidant properties and can suppress 6-OHDA-induced intracellular ROS production. Furthermore, orexin-A has been found to exert a protective effect against cell apoptosis by decreasing caspase-3 activation and the Bax/Bcl2 ratio (26). Therefore, it is plausible that the analgesic effects of orexin-A on diabetic neuropathy may be mediated, at least in part, through its ability to prevent oxidative stress and neuronal apoptosis. On the other hand, the vlPAG is a key nucleus within the descending pain modulatory system, where the tonic activity of GABAergic neurons leads to antinociception via the inhibition of nociceptive transmission. It is conceivable that orexin-A may produce analgesia, at least in part, by activating postsynaptic orexin receptors on vlPAG neurons, thereby stimulating the synthesis of the endocannabinoid 2-arachidonoylglycerol (2-AG) through a Gq protein-coupled PLC–DAGLα

signaling cascade. This, in turn, could result in the retrograde inhibition of GABA release (i.e., disinhibition) within the vlPAG, ultimately culminating in an attenuation of pain responses (11). However, an important fact from our results is that the analgesic effects were sustained for a longer duration in the healthy animals compared to the more transient effects observed in the diabetic animals. Specifically, in the diabetic group, the anti-nociceptive actions of PAG orexin administration were limited to the initial time points following administration. These findings suggest that the activation of orexin receptors within the PAG can powerfully modulate pain processing, at least acutely, in both normal and pathological pain states. However, the temporal dynamics of this orexinergic modulation may be altered in the context of diabetic neuropathy. In the present study, orexin-A was administered into the PAG as a single, one-time injection. This may explain the more transient effects observed in the diabetic animals. It is possible that repeated or continuous delivery of orexin-A into the PAG may be necessary to maintain the analgesic benefits over a prolonged period in the context of neuropathic pain related to diabetes.

CONCLUSION

Collectively, these findings highlight the pivotal role of orexinergic neurotransmission, particularly within the PAG, in the top-down modulation of pain processing under both normal and pathological conditions. Further elucidation of the precise neuroanatomical and neurochemical mechanisms through which orexin exerts its analgesic effects within the descending pain modulatory network holds promise for the development of novel therapeutic interventions targeting debilitating chronic pain states.

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DECLARATIONS

Authors have no conflict of interest to declare.

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