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Activation of Orexin-A and Capsaicin Receptors in the Periaqueductal Gray is Ineffective in Reducing Mechanical Allodynia and Thermal Hyperalgesia in Healthy and Diabetic Rats

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Authors

Talieh Shirafkan[1]

Alireza Komaki[2,3]

Abdolrahman Sarihi[2,3,*]

1. *Department of Biology, Faculty of Basic Sciences, Islamic Azad University of Hamadan, Iran*
2. Neurophysiology Research Center, Hamadan University of Medical Sciences, Hamadan, Iran
3. Department of Neuroscience, School of Sciences and Advanced Technology in Medicine, Hamadan University of Medical Sciences, Hamadan, Iran

***Corresponding author:**

Abdolrahman Sarihi

Department of Neuroscience, School of Sciences and Advanced Technology in Medicine, Hamadan University of Medical Sciences, Hamadan, Iran, Postal code: 6517838736

E-mail: asarihi@yahoo.com

sarihi@umsha.ac.ir

ABSTRACT

Introduction: Up to now, numerous neural circuits within the central nervous system have been identified as participants in the modulation of pain. Among these, the ventrolateral periaqueductal gray (vlPAG) region of the midbrain stands out as a crucial component of the pain modulation network at supraspinal levels. Its involvement has been extensively characterized across various animal pain models. In this research, we focused on investigating the roles of orexin A and capsaicin receptors within this region in mediating antinociceptive responses.

Methods: In this study, male Wistar rats were utilized to explore the antinociceptive effects of administering orexin-A and capsaicin directly into the ventrolateral periaqueductal gray (vlPAG) region of the midbrain. The substances were administered both separately and in combination. Then, their antinociceptive effects were assessed using the von Frey and hot plate tests post-injection. The experiments compared the outcomes of intra-vlPAG drug administration in both healthy control and diabetic animal models.

Results: Microinjections of orexin-A and capsaicin, whether administered individually or in combination directly into the PAG, failed to produce anti-nociceptive effects on mechanical allodynia or thermal hyperalgesia in both healthy and diabetic rats.

Conclusion: Acute microinjections of orexin-A and capsaicin did not produce significant anti-nociceptive effects in either healthy or kindled animal groups. To draw more accurate conclusions, it is recommended to investigate the long-term effects of these compounds as well.

Keywords: Pain, Mechanical allodynia, thermal hyperalgesia, Orexin A receptor, Capsaicin receptor, Ventrolateral periaqueductal gray, Diabetic neuropathy.

INTRODUCTION

Temporary changes in the nervous system following tissue injury are generally adaptive, serving to trigger acute pain as a warning signal to prevent further damage. Pain, as a natural response to harmful external and internal stimuli, plays a crucial role in maintaining vigilance due to its unpleasant nature. However, when the nervous system itself is damaged, these temporary changes can become chronic, leading to neuropathic pain (NP) syndromes (1). In such cases, pain loses its protective function and instead contributes to debilitating and health-compromising conditions (2). Diabetic neuropathy pain (DNP) exemplifies this, as it can present with severe and prolonged sensitivity to harmful stimuli (hyperalgesia) and an exaggerated response to normally harmless

stimuli (allodynia) (3-5). The underlying mechanisms of DNP in patients and animal models remain elusive.

Orexin, also referred to as hypocretin, is synthesized in the hypothalamus, with orexinergic neurons extending into various regions of the brain. There are two identified isoforms: orexin-A and orexin-B. Orexin-A, through its receptor activation, is involved in numerous physiological functions, including the regulation of sleep and wakefulness, energy metabolism, feeding behavior, learning, and memory (6, 7).

Conversely, capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide, C18H27NO3) is a volatile and pungent compound extracted from peppers, known for its burning and irritating effects. Recent research has highlighted capsaicin's beneficial properties, such as its antioxidant and anti-inflammatory effects, its ability to lower blood pressure, promote weight loss, alleviate pain, and potentially prevent cancer (8-10).

This study aimed to explore the potential of orexin A and capsaicin in mitigating mechanical allodynia and thermal hyperalgesia within the context of diabetes. Utilizing a rodent model of streptozotocin-induced diabetes, we sought to elucidate the effects of direct administration of orexin A and capsaicin on pain sensitivity in this disease state, which is marked by peripheral nerve dysfunction.

MATERIALS AND METHODS

Experimental subjects and ethical standards

The research team utilized a total of 24 male Wistar rats, each weighing between 250 to 300 g. These animals were sourced from the renowned Pasteur Institute of Iran, located in Tehran. The rats were housed together in groups within a controlled environment throughout the duration of the experiment. Stringent measures were taken to maintain optimal conditions for the animals' well-being. Specifically, the rats were kept on a carefully regulated 12-hour light-dark cycle. The lights in the housing area were programmed to turn on at 7:00 AM and turn off at 7:00 PM each day, mimicking a natural day-night pattern. The ambient temperature of the environment was also meticulously monitored and maintained between 22 to 25 degrees Celsius, providing a comfortable and stable thermal setting for the subjects.

Importantly, the rats were given unrestricted access to a standard rodent chow diet as well as clean, sterile drinking water at all times. This ensured the animals could freely consume food and hydrate themselves as needed during the course of the study.

All experiments and procedures involving these Wistar rats were conducted in strict accordance with the guidelines outlined in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). This comprehensive set of protocols helped guarantee the humane treatment and ethical use of the animal subjects throughout the research.

Surgical technique and recovery period

The surgical intervention began by inducing a deep state of anesthesia in the Wistar rats. This was achieved through an intraperitoneal injection of sodium pentobarbital, a potent anesthetic agent, at a dosage of 10 milligrams per kilogram of the animal's body weight. To further minimize any potential pain and bleeding during the procedure, a local anesthetic solution was also administered. This solution contained a combination of lidocaine, a numbing agent, and epinephrine, a vasoconstrictor, in a 0.2 milliliter volume. The researchers carefully injected this anesthetic mixture around the intended surgical area on the rat's skull. With the rats suitably anesthetized and the surgical site numbed, the animals were then positioned within a specialized stereotaxic apparatus. This device, manufactured by Stoelting in the United States, allowed for the precise and controlled placement of the surgical instruments during the procedure. Next, a linear incision was made along the midline of the scalp, exposing the surface of the skull. The researchers identified and carefully cleaned two distinct cranial landmarks, known as the bregma and lambda, which served as anatomical reference points. Using these landmarks as guides, the researchers then implanted a stainless steel guide cannula, targeting a specific region of the midbrain called the ventrolateral periaqueductal gray (vlPAG) area. The stereotaxic coordinates

relative to the bregma were: anterior-posterior = -7.8 mm, medial-lateral = +0.8 mm, and dorsal-ventral = 6.4 mm from dura, as per the standardized rat brain atlas (11). To securely anchor the implanted guide cannula in place, the researchers utilized two additional stainless steel screws and applied dental cement over the entire assembly.

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

Diabetes induction

The researchers chose to induce a diabetic condition in the experimental group of Wistar rats through a single intraperitoneal (injected into the abdominal cavity) administration of the pharmacological agent streptozotocin (STZ). This drug was freshly prepared and given at a dose of 60 mg/kg of the animal's body weight. Three days after the STZ injection, the researchers obtained a small blood sample from each rat via a prick of the tail vein. These blood samples were then analyzed using a digital glucometer, specifically the AVAN AGM01® model from Iran. The purpose of this analysis was to measure the concentration of glucose present in the rats' blood. Any rats that displayed blood glucose levels exceeding 250 mg/dl were subsequently classified as being in a diabetic state. The day on which this hyperglycemic condition was confirmed marked the official starting point of the research study. In contrast, the control group of Wistar rats received an equivalent volume of the citrate buffer solution, which served as the vehicle or carrier for the STZ in the experimental group.

Throughout the duration of the study, the researchers continued to monitor and record the body weights and blood glucose levels of all the rats, both at the beginning and the end of the experimental period. This allowed the researchers to track the progression of the induced diabetic condition in the experimental group compared to the control group.

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.

Capsaicin: Capsaicin was also obtained from Sigma–Aldrich Co. (USA). Capsaicin was prepared by dissolving it in 10% ethanol.

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 drugs (capsaicin; 10 nmol/ 0.5 µl or orexin A; 10 nmol/ 0.5 µl) or the vehicle (10% ethanol or 0.9 % NaCl). 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.

Behavioral tests

Mechanical allodynia

To evaluate mechanical allodynia, the paw withdrawal threshold (PWT) was determined by applying a series of calibrated von Frey filaments to the plantar surface of the hind paw. Following a 30-minute acclimation period, von Frey filaments with progressively increasing bending forces (measured in grams) were applied to the hind paw. Each filament was tested five times at 10-second intervals. The assessment commenced with the lightest filament (0.008 g) and continued with filaments of greater force until a positive response was observed. A positive response was characterized by the animal withdrawing its paw in at least three out of the five stimulations. A filament with a force of 60 g was used as the upper limit. A 5-minute rest period was allowed between the applications of different filament strengths.

Thermal hyperalgesia

Thermal hyperalgesia was evaluated by measuring the paw withdrawal latency (PWL) in response to the application of noxious radiant heat to the hind paw, utilizing a hot plate apparatus (Tahgos teb, Iran). The rats were placed in a transparent plexiglass cage and allowed a 30-minute acclimation period prior to testing. The PWL was determined by exposing the plantar surface of the hind paw to a focused radiant heat source, and the time taken for the animal to lift or lick its hind paw was recorded as the PWL. To prevent potential tissue damage, an automatic cut-off time of 30 seconds was implemented. The thermal stimulus was applied three times, with 5-minute intervals between each application, and the average PWL values were reported.

Experimental design

The animals were randomly allocated to control and diabetic groups. Each experimental groups were divided into four subgroups, including vehicle-treated group named as control group, orexin-A-treated group, capsaicin-treated group, and orexin-A + capsaicin-treated group. This experiment investigated the effects of intra-vlPAG orexin-A and/or capsaicin on mechanical allodynia and thermal hyperalgesia, either in separate groups or in combination.

Histology

Following the completion of the behavioral tests, 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 one-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 and capsaicin microinjections into the vlPAG show no effect on mechanical allodynia in both healthy and diabetic rats

The statistical analysis revealed no significant difference in von Frey threshold between the experimental groups in healthy and also diabetic rats (Fig 1 & 2).

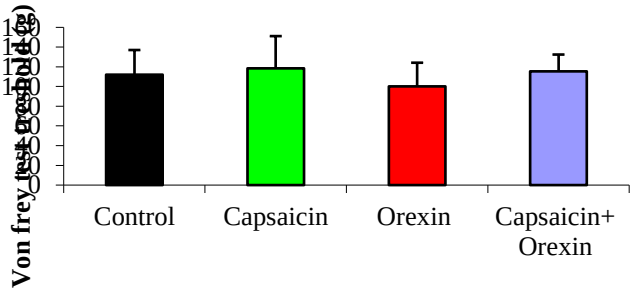

Fig. 1. The bar graph illustrates the impact of microinjection of orexin A and capsaicin into the ventrolateral periaqueductal gray (vlPAG) region on tactile pain threshold in healthy animals (n=6). Data represent mean ± SEM.

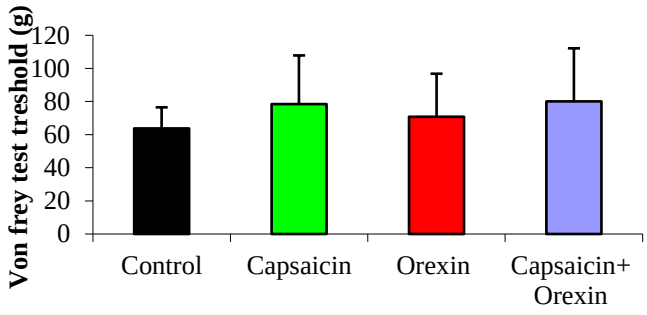

Fig. 2. The bar graph illustrates the impact of microinjection of orexin A and capsaicin into the ventrolateral periaqueductal gray (vlPAG) region on tactile allodynia in diabetic animals (n=6). Data represent mean ± SEM.

Ineffectiveness of orexin A and capsaicin microinjections into the vlPAG on thermal hyperalgesia in both healthy and diabetic rats

Statistical analysis indicated that there was no significant difference in hot plate latency among the experimental groups, regardless of whether the rats were healthy or diabetic (Fig 3 & 4).

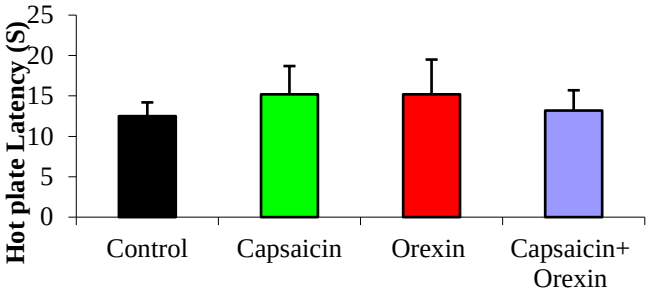

Fig. 3. The bar graph illustrates the impact of microinjection of orexin A and capsaicin into the ventrolateral periaqueductal gray (vlPAG) region on hot plate latency in healthy animals (n=6). Data represent mean ± SEM.

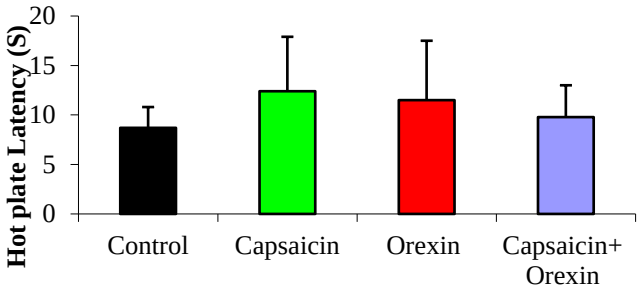

Fig. 4. The bar graph illustrates the impact of microinjection of orexin A and capsaicin into the ventrolateral periaqueductal gray (vlPAG) region on hot plate latency in diabetic animals (n=6). Data represent mean ± SEM.

DISCUSSION

In this study, we clearly demonstrated that microinjections of orexin-A and capsaicin, whether administered separately or together directly into the PAG, did not produce anti-nociceptive effects on mechanical allodynia or thermal hyperalgesia in both healthy and diabetic rats. These findings contrast with previous research suggesting that capsaicin and orexin have been effective as adjunct pharmacological agents in pain management. For instance, prior studies have shown that capsaicin treatment is effective for various painful conditions, including complex regional pain syndromes, neuropathic pain (12, 13), postsurgical neuropathic pain (14, 15), post-herpetic neuralgia (16, 17), and painful diabetic peripheral neuropathy (18, 19). Additionally, repeated nasal capsaicin use has been reported to prevent cluster headache attacks (20). In humans, topical capsaicin (0.075%) applied four times daily for three weeks leads to the degeneration of skin nerve fibers, thereby reducing sensitivity to cold and tactile stimuli, but not to heat and mechanical stimuli (21).

Furthermore, several animal studies have indicated that orexin-A induces thermal analgesia. In the hot-plate test, activation of the Orx1 receptor in the periaqueductal gray of rats produced antinociception, which was blocked by either the Orx1 receptor antagonist SB334867 or the CB1 receptor antagonist AM 251 (22). Additionally, blocking the Orx1 receptor in the vlPAG has been associated with decreased tail-flick latency following carbachol-induced antinociception (23). However, as previously mentioned, our results indicate that microinjections of orexin or capsaicin do not exhibit analgesic effects in healthy and diabetic animals. This lack of effect may be attributed to the dosage used or the frequency of drug administration.

CONCLUSION

Given that our study did not demonstrate significant anti-nociceptive effects on mechanical allodynia and thermal hyperalgesia with the activation of orexin and capsaicin receptors in either healthy or diabetic rats, it is recommended to use higher doses of these compounds for a longer duration to obtain more accurate results. Additionally, investigating the precise molecular mechanisms involved is suggested.

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DECLARATIONS

Authors have no conflict of interest to declare.

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