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Repeated injections of orexin-A developed behavioral tolerance to its analgesic effects in rats

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ABSTRACT

Objective(s): Reduction of pharmacological effectiveness or tolerance appears following repeated administration of many analgesic drugs. We investigated tolerance to anti-nociceptive effects of orexin-A, an endogenous potent analgesic peptide using the hot-plate test.
Materials and Methods: Orexin-A was microinjected ICV (intracerebroventricular) with an interval of 12 hr for 7 continuous days and its anti-nociceptive responses were measured on days 1, 4 and 7 using the hot-plate test following the first day of administration. Orexin-A was used at a dose of 100 pmol to induce analgesic effects.
Results: ICV administration of orexin-A produced an effective anti-nociception on the first day of experiment as measured by hot-plate 5, 15, and 30 min after the injection, in comparison with both baselines (hot-plate test one day before the beginning of orexin-A administration and control, saline-administrated group). However, repeated administration of orexin-A on the following days revealed a significant reduction in this analgesic effect during day 4 to day 7. However, to rule out any associative tolerance resulting from learning related to experimental procedures and/or environmental cues, a single injection of orexin-A was administrated to animals of control group (which were receiving saline during 7 days of experiments) and the analgesic effect was observed.
Conclusion: These results, for the first time, indicated the appearance of tolerance to anti-nociceptive effects of orexin-A, following repeated administrations of this agent.

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Introduction

Opioid derivatives, particularly μ-opioid receptor agonists such as morphine, are the most effective and widely prescribed therapy for treatment of moderate-to-severe pain (1, 2). However, when opioids are repeatedly used for pain relief , the analgesic effects of these agents are accompanied by unwanted side effects such as physical dependence and hence, must be administered in escalating doses due to rapid development of tolerance to their analgesic actions (3-5). Over the past decades, there has been an intensive search for analgesic agents without these side effects.

Orexin-A and B, a well-known pair of hypothalamic peptides, are identified as regulators of feeding behaviors and sleep-wake cycle (6, 7). They are also involved in responsiveness to both pain and stressful stimuli (8). Orexins act at least on two subtypes of G-protein coupled receptors known as orexin receptor-1 and 2. Orexin projections and receptors expression found in multiple brain regions are involved in pain modulation such as ventral tegmental area (VTA), nucleus accumbens, hippocampus, hypothalamic, dorsal and medial raphe, locus coeruleus (LC), periaqueductal gray (PAG), and reticular formation (9, 10).

Previous behavioral researches have shown analgesic effects of orexin-A (11-17). These studies have indicated that the efficacy of orexin-A as an analgesic agent is similar to that of morphine in 50 ˚C hot-plate test (13). In addition, evidences emphasize that orexin receptor-1 is involved in responsiveness to both pain and stressful stimuli; therefore, it may have a key role in stress-induced anti-nocecption (SIA) (18, 19). Orexin mechanisms of anti-nociception have not yet been clarified, but as orexin receptor-1 is expressed in both brain and spinal cord, it is proposed that both mechanisms contribute to the anti-nociceptive in both brain and spinal cord, it is proposed that both mechanisms

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contribute to the anti-nociceptive effects of orexins (13, 20, 21). Recently, it has been has shown that microinjection of orexin-A into the periaqueductal gray (PAG) produces analgesic effect (12).

Tolerance, as a result of mal-plasticity in the nervous system mechanisms, appears with a decrease in responsiveness and increase in demand for drug (22). Repeated administration of some analgesic drugs causes tolerance to anti-nociceptive effect of these substances (23), as a clinically undesirable outcome. Therefore, our attention was attracted by the question that whether repeated administration of orexin-A would induce tolerance to anti-nociceptive effect of this peptide.

Materials and Methods

In all experiments, adult male Sprague-Dawley rats (200-250 g) purchased from Razi Institute, Karaj, Iran were used. Animals were housed in an environment with 12 hr/12hr light/dark cycle at room temperature (22±2 °C). All research and animal care procedures were done according to the international guidelines on the use of laboratory animals.

Stereotaxic surgery and intracerebroventricular microinjection

Animals were anaesthetized with a mixture of ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively; IP injection) and placed in a stereotaxic apparatus (Stoelting, USA). To perform direct ICV administrations of chemicals, the dura was exposed with drilling the skull at an appropriate and previously labeled site just above the lateral ventricle (LV, coordination: AP, -0.9 mm; L, -1.8 mm; V, -3.8 mm all from the bregma) according to the rat brain atlas (22). Following removal of the dura, a 23-gauge, stainless steel guide cannula was implanted just 2 mm above the right side of the LV. The cannula was anchored in the skull on two stainless steel screws using dental cement. After ligation of the incision site, animals were kept individually for recovery.

A 30-gauge needle (protruded 2 mm beyond the guide cannula to reach the right LV and connected through a polyethylene tube to a 10 µl Hamilton syringe) mounted on a stereotactic micromanipulator, was used to inject chemicals. A volume of 5 µl of orexin-A (25, 100, and 200 pmol) in sterile 0.9% saline (pH 7.4) was microinjected ICV (at a rate of 2 µl/min) to determine the optimum dose for analgesic effects. Control animals were injected with the same volume of sterile saline.

Hot-plate test

A hot-plate apparatus (Analgesia meter IITC, Life science, USA) was used to measure the time latencies to pain. Animals were placed in an acrylic box (22.5× 22.5 cm in diameter) on the heated surface, and the time to lick paws or jump, was recorded as the response latency. A 50 sec cut-off was used to prevent tissue damage. The response latency of all animals was measured one day before the beginning of drug microinjections to provide baseline response for each group. Temperature of hot-plate apparatus was set and held at 52 ± 1 ˚C for all experiments (23). The animals presenting training latencies higher than 30 sec were excluded. Animals were microinjected ICV with orexin-A or saline and subjected to the hot-plate test after 5, 15, 30, and 60 min. The dose of orexin-A was selected based on the data from the dose-response experiments.

Experimental design

Animals were divided into two experimental groups (n = 8 in each group). In group 1, orexin-A was given unilaterally (ICV) for six days (twice per day with 12 hr intervals). In group 2, saline was given (ICV) as orexin-A. To prevent associative learning during the hot-plate test, the analgesic effects of the chemicals were evaluated on days 1, 4, and 7 using hot-plate test at 5, 15, 30, and 60 min following morning administrations. The microinjection procedure might cause a learning process which results in progressive shortening of the jumping reaction or licking behavior in hot-plate test. By this, we tried to prevent association learning that might happen between microinjection of drug and affect jumping reaction or licking behavior in hot-plate test.

Body weight

In both groups, the body weight was measured every two days, from the day of baseline test until the end of the experiments.

Histological verification

At the end of the experiments, all animals received 5 µl of pontamine sky blue (0.2%) ICV through the cannula and were later anaesthetized with ketamine overdose. Rats were initially perfused intracardially with 100-150 ml of PBS solution and later with equal volume of 4% formalin solution. Subsequently, the brain was removed and sectioned. Only the data from rats whose diffusion and microinjection site were confirmed in the LV (19), were included. In all experiments, orexin-A was microinjected into the LV.

Data analysis

Data were expressed as mean ± SEM. After testing the data for their normal distribution (Kolmogorov-Smirnov), they were analyzed using repeated-measures ANOVA or one-way ANOVA (by Dunnett's test) for comparison between groups. Statistically, significant were defined when $P<0.05$.

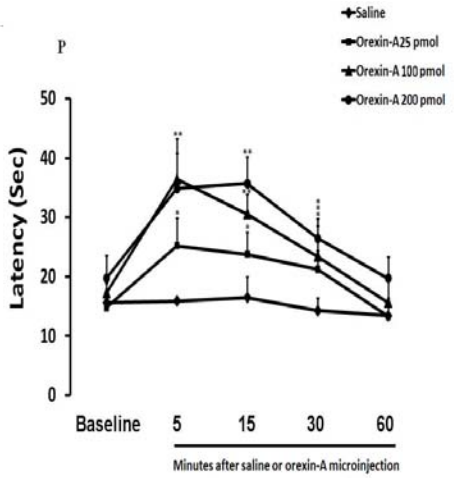

Figure 1. Dose-response test. Curves showing the antinociceptive effect produced by three different concentrations (25, 100 and 200 pmol) of orexin-A when microinjected into the LV. Antinociceptive responses were measured at 5, 15, 30, and 60 min after microinjection. Values demonstrated as mean±SEM and n=8 for each dose. * P<0.05; ** P<0.001 and *** P<0.01 in comparison with vehicle group

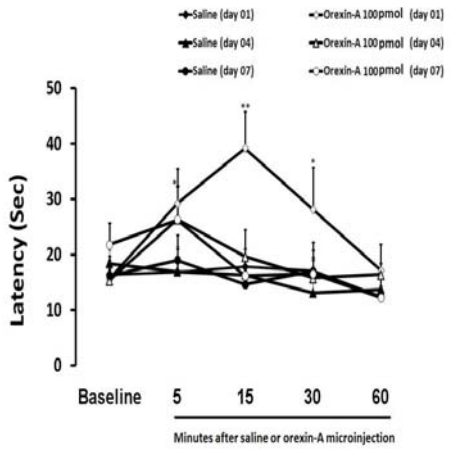

Figure 2. Chronic administration of orexin-A induced tolerance to its antinociceptive effect. As seen in this figure, saline microinjection on continuous days had no effects on latency of responses on different days of hot-plate test. Orexin-A (100 pmol) microinjections into the LV showed a potent analgesic effect on day 2 when measured with hot plate at 5, 15, and 30 min but not 60 min (P<0.05) after its administration however, the repeated application of orexin-A on following days demonstrated a significant decline in its analgesic effect when measured on days 4 and 7 by hot plate. Data presented as mean ± SEM and n=8 for each group. * P<0.05 and ** P<0.001 in comparison with vehicle group

Results

Dose-response test

The three doses of orexin-A (25, 100, and 200 pmol) selected for dose-response test showed anti-nociceptive effect in hot-plate test, 5, 15, and 30 min after microinjection. As seen in Figure 1, the lowest dose of orexin-A (25 pmol) produced anti-nociception for 30 min (P<0.05). In addition, microinjection of the two other doses of orexin-A (100 and 200 pmol) showed even more marked analgesic effect whereas the difference was statistically insignificant (P>0.05). However, it seems that the highest dose of orexin-A (200 pmol) produced a longer anti-nociception, 60 min after microinjection (Figure 1).

Chronic orexin-A induced tolerance to its anti-nociceptive effect

According to Figure 2, the hot-plate latencies increased significantly on the first day, 5, 15, and 30 min after microinjection of orexin-A (100 pmol) compared to the baseline and saline-microinjected animals (P<0.05 for 5 and 30 min and P<0.01 for 15 min). According to the experiment, maximum anti-nociception occurred 15 min after the administration of orexin-A indicating a potent analgesic effect for orexin-A. However, 60 min after orexin-A microinjections, the latency of responses was similar to baseline and control groups. Furthermore, when orexin-A was administrated on the following days (twice per day) and the latency of responses were measured on days 4 and 7, analgesic effect significantly decreased toward the baseline and saline groups (P>0.05) (Figure 2) indicating the occurrence of tolerance following chronic orexin-A.

Chronic orexin-A caused weight loss

As demonstrated in Figure 3, a significant difference in body weight was observed between the control group and animals that were treated with orexin-A in a chronic manner (P<0.01). Body weight was controlled daily along the experiments and the differences in body weight were measured between the day of baseline test and the day of final microinjection. While saline-microinjected animals showed an increase in their body weights (3.25±2.69 g), orexin-A-administrated animals showed weight loss (9.9±2.29 g).

Discussion

In the current research, for the first time, we provide evidence that chronic administration of orexin-A into the lateral ventricle produced tolerance to the anti-nociceptive effect of this peptide. Several studies have shown anti-nociceptive effects of orexins in various animal models of pain (12, 14, 21, 24, 25). Herein, our results also confirmed the analgesic activity of orexin-A. Anti-nociceptive effect of orexin-A, but not orexin-B, has been described to be as pronounced as morphine (13). However, a clinically unfavorable problem with the chronic administration of opioids is development of tolerance to their analgesic actions (22). Gene expression studies have shown that orexin receptors are widely distributed in both brain and spinal cord (20). Central administration of orexin-A agonist into the brain and spinal cord have revealed significant

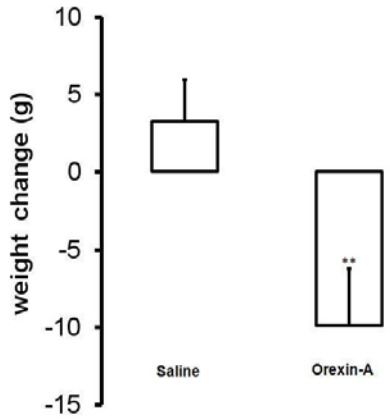

Figure 3. Loss of body weight resulted from chronic orexin-A. Body weight showed an increase in saline-administrated animals whereas the orexin-A-microinjected animals revealed a significant decrease in body weight (P<0.05). Body weight measurement was followed daily during the experiments; however, only the difference between the day of baseline test and the last day of microinjection is shown here. ** P<0.001 in comparison with vehicle group

pain relief in various animal models of pain (12, 16, 20). This effect seems to be opioid-independent and mainly mediated through OX1 receptors (21, 25). However, in all of these reports, orexin was given in an acute manner and thus, it is unclear whether chronic administrations of these peptides show similar effect.

To find an appropriate dose of orexin-A with anti-nociceptive effect following ICV microinjection, a dose-response test was designed. Our results showed that maximum analgesic action appears following administration of 200 pmol orexin; however, as the same effect was almost produced by 100 pmol of orexin, this amount was selected as the optimum dose for other experiments. Some studies have investigated systemic anti-nociceptive effect of orexin-A in different animal models of pain (summarized table in Chiou et al., 2010) (26) and most of them used orexin-A at doses from 0.1 nmol to 1 nmol for ICV (5 µl) or intrathecal (IT, 10 µl) injection. Moreover, a similar amount of orexin-A (0.5 nmol) was microinjected (ICV) for examining the effects of this agent on food intake and body weight (27).

There is a controversy over the effects of orexins on body weight. It has been reported that ICV microinjection of orexin-A (0.5nmol/h for 7 days) has no effect on body weight despite an increase in daytime food intake (28). We observed a significant weight loss in animals treated with orexin-A for 7 days as compared to saline group. In our study, animals weight was measured daily as they were under microinjection of orexin or saline twice a day. The microinjection protocol used by Yamanaka *et al* (28) was different from that we used in this study which may explain the discrepancies in results between these two studies. As revealed by our data, repeated administration of orexin-A not only prevented weight increase, but also produced weight loss. Novak and Levine (27) have reported the occurrence of weight loss following chronic orexin-A microinjection into the paraventricular nucleus (PVN). Although, they applied orexin-A into a paraventricular nucleus at a different dose, their results are similar to ours. It has been reported that orexins increase the food intake (27) and also affect the spontaneous physical activity and wakefulness; therefore, a weight loss due to negative energy balance may be expected (29).

Although, repeated administration of orexin-A produced a significant decrease in anti-nociceptive response, pharmacological tolerance was observed. In addition, changes in arousal, cardiovascular activation, hyperlocomotion and alteration of homeostasis following chronic administration of orexin-A might occur and become confounding factors for the hot-plate response, although we disregarded them but these factors should be considered in future studies.

It has been suggested that the opioid tolerance mainly occurs through adaptation at the level of opioid receptor itself. Other studies, however, have demonstrated additional adaptation mechanisms appearing downstream from the opioids receptor and involving the activation of NMDA receptors, translocation and activation of protein kinases, uncoupling of opioids receptors from G proteins and adenylate cyclases, supersensitivity of the adenylyl cyclase system, and downregulation and internalization of opioids receptors (1, 2, 5, 30) Gintzler and Chakrabarti described a shift from Gi inhibitory to the Gβγ stimulatory in opioids tolerance (3). Previous investigations have shown that the orexin-1 receptors mainly couple to the Gq/11 subclass of heterotrimeric G proteins whereas the orexin-1 receptors pair with Gq/11, Gi/o, and Gs (31). Also, it has been reported that the protein kinases such as PKC, PKA, and CamKII mediate the effects of orexins in cells (32, 33). According to the previous studies, the cellular signal transduction mechanisms of orexins and opioids have many similarities. Therefore, same cellular mechanisms might be responsible for appearance of tolerance to analgesic action of orexin following chronic use; however, further in-depth studies are needed to investigate the cellular mechanisms.

In all previous investigations on the analgesic action of orexins, a single injection of these peptides was applied which led to occurrence of a potent analgesic effect that was comparable with morphine, but this is assumed to be independent of endogenous opioidergic system (14, 21, 25). However, as the orexin central action is mediated via PAG and the descending pain control system, it could be speculated that the opioidergic system might be

involved in both analgesic action and tolerance produced by orexins injection. Prior to the current study, there is no available report in the literature regarding the chronic administration of these peptides and their analgesic effect. Considering this potent effect of orexin, these peptides might further support the development of orexin-1 agonists for pain treatment in clinical settings. It is noteworthy that discovery of analgesic agents with zero to little tolerance following chronic administration is of vital importance.

Conclusion

Herein, we centrally administered orexin-A for 7 continuous days and interestingly a reduction in its effectiveness after the 4th day of microinjection was observed. Further investigations are needed to find the mechanism(s) of this tolerance produced by the repeated injection of orexin-A and if such phenomenon occurs for the other effects of orexins then, the next important question will be whether the development of tolerance could be avoidable.

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