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Neuroprotective and antihyperalgesic effects of orexin-A in rats with painful diabetic neuropathy

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ABSTRACT

Aim of study: Diabetes mellitus is related to the development of neuronal tissue injury in different peripheral and central nervous system regions. A common complication of diabetes is painful diabetic peripheral neuropathy (PDN). We have studied the neuroprotective and anti-nociceptive properties of neuropeptide orexin-A in an animal experimental model of diabetic neuropathy.

Methods: All experiments were carried out on male Wistar rats (220-250 g). Diabetes was induced by a single intraperitoneal injection of 55 mg/kg (i.p.) streptozotocin (STZ). Orexin-A was chronically administrated into the implanted intrathecal catheter (0.6, 2.5 and 5 nM/L, daily, 4 weeks). The tail-flick and rotarod treadmill tests were used to evaluate the nociceptive threshold and motor coordination of these diabetic rats, respectively. Cleaved caspase-3, Bax, Bcl2 and the Bax/Bcl-2 ratio, as the biochemical indicators of apoptosis, were investigated in the dorsal half of the lumbar spinal cord tissue by western blotting method.

Results: Treatment of the diabetic rats with orexin-A (5 nM/L) significantly attenuated the hyperalgesia and motor deficit in diabetic animals. Furthermore, orexin-A (5 nM/L) administration suppressed pro-apoptotic cleaved caspase-3 and Bax proteins. Also, orexin-A (5 nM/L) reduced the expression of Bax/Bcl-2 ratio in spinal cord dorsal half of rats with PDN.

Conclusions: Altogether our data suggest that the orexin-A has anti-hyperalgesic and neuroprotective effects in rats with PDN. Cellular mechanisms underlying the observed effects may, at least partially, be related to reducing the neuronal apoptosis.

1. Introduction

Diabetes mellitus (DM) is one of the largest epidemics the world has faced, both in developed and developing nations (Zimmet et al., 2016). Diabetic neuropathy (DN) develops a background of hyperglycemia and an entangled metabolic imbalance (Yang et al., 2014). One of the most elusive symptoms in diabetic neuropathy is pain which characterized by mechanical and thermal hyperalgesia (Kaeidi et al., 2011). Additionally, several investigations showed motor deficits in human and animal subjects with DN (Esmaeili Mahani and Kaeidi, 2012; Gutierrez et al., 2001; Hajializadeh et al., 2014; Rasoulian et al., 2018; Said et al., 2008).

It has been shown that hyperglycemia is the main cause of nervous system damage (Kaeidi et al., 2011). Furthermore, several studies revealed that, high glucose situation leads to apoptosis and neural dysfunction in several studies (Kaeidi et al., 2011; Kaeidi et al., 2013; Hajializadeh et al., 2014; Kaeidi et al., 2015; Saberi Firouzi et al., 2018) However, the degree of apoptosis in neuronal tissues plays a critical role in the pain behavior (Sekiguchi et al., 2009).

In the lateral and dorsal hypothalamus, the endogenous neuropeptide orexin (or hypocretin) is produced from the precursor pre-pro-orexin (Chrobok et al., 2017). The orexin system contains two G-protein

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coupled receptors including orexin-1 (Orx1) and the orexin-2 (Orx2) (Lee et al., 2016). Orexin-A has equal affinity for Orx1 and Orx2 receptors, while orexin-B has 10-fold higher affinity for Orx2 than Orx1 receptors (Boss and Roch, 2015; Toyama et al., 2017). In addition, detailed mapping of orexin receptor mRNA distribution has shown that the two types of orexin receptors are distributed throughout the rat brain, with different expression patterns (Hervieu et al., 2001; Lu et al., 2000; Trivedi et al., 1998). Also, both orexin-A and Orx1 receptors have been found in spinal cord dorsal root ganglion cells (Bingham et al., 2001). Both of the Orx receptors have been implicated in numerous physiological and biological functions. These mainly include energy homeostasis (Coborn et al., 2017), sleep regulation (Kukkonen, 2012), feeding (Yamada et al., 2000), opioid dependence and tolerance (Ahmadi-Soleimani et al., 2017; Ahmadi-Soleimani et al., 2014; Ghaemi-Jandabi et al., 2017), neuroendocrine function (Toyama et al., 2017) and pain modulation (Kargar et al., 2015; Soleimani et al., 2015). Orexinergic neurons send their projections to several regions of the central nervous system that participate in analgesia, such as the raphe nucleus, locus coeruleus nucleus and midbrain periaqueductal gray (Peyron et al., 1998). These regions are known to play main roles in the modulation of the pain (Ossipov et al., 2010). It has been revealed that orexinergic neurons also directly send their projections to the spinal dorsal horn (Van Den Pol, 1999). In the spinal cord dorsal horn, orexin containing fibers are predominantly present in the superficial laminae associated with nociceptive processing (Date et al., 2000; Grudt et al., 2002; Hervieu et al., 2001). It has been shown that orexins have antinociceptive effects in the brain and spinal cord in different types of pains including mechanical, chemical, thermal induced nociceptions and nociceptin-induced behavioral responses (Toyama et al., 2017; Mobarakeh et al., 2005; Razavi and Hosseinzadeh, 2017). Also, numerous findings have shown that orexin-A has neuroprotective effects in in vivo and in vitro studies (Esmaeili-Mahani et al., 2013; Yuan et al., 2011; Feng et al., 2014; Davies et al., 2015; Yan et al., 2008). Furthermore, it has been shown that orexin-A has antiapoptotic effects in several investigations (Butterick et al., 2012; Duffy et al., 2016; Shu et al., 2017; Wang et al., 2018). So, the aim of this study is to examine the possible neuroprotective and antinociceptive effects of Orexin-A by reducting the hyperglycemia-induced neuronal apoptosis in streptozotocin-induced diabetic rats with PDN. We evaluated the thermal nociception, motor function, and some molecular apoptotic markers to investigate the underlying mechanisms.

2. Materials and methods

2.1. The main chemical and materials

Orexin-A from Tocris Bioscience (USA) was dissolved in sterile normal saline (NS). Streptozotocin (STZ) was obtained from Biosera Co. (East Sussex, UK). Primary monoclonal anti-caspase-3, anti-Bax, anti-Bcl2, anti-beta-actin antibody, and secondary monoclonal anti-rabbit monoclonal antibodies were purchased from Abcam (USA). PVDF membranes and Lumi-Film chemiluminescent detection film were purchased from Roche (USA). Leupeptin, phenylmethylsulfonyl fluoride, sodium orthovanadate, aprotinin, acrylamide amid and bisacrylamide were obtained from Sigma (USA).

2.2. Animals

The experiments were carried out on male Wistar rats, weighing 220–250 g, that were housed four per cage under a 12 h light/dark cycle in a room with controlled temperature (22 ± 1 °C). Food and water were available ad libitum. Animals were handled daily (between 9:00 and 11:00 A.M) 5 days before the experiment in order to adapt them to manipulation and minimize non-specific stress responses. The rats were randomly allocated into seven experimental groups. All experimental procedures were carried out in accordance with the guidelines for the care and use of laboratory animals in the Rafsanjan University of Medical Sciences (Ethic code: IR.RUMS.REC.1395.059) and the Principles of Laboratory Animal Care (NIH publication #85–23, revised in 1985).

2.3. Experimental groups

The rats were separated into 7 experimental groups (6–8 animals per group): 1) Control group. Intact animals (without any treatment or surgical procedure). 2) Sham group. The non-diabetic rats with surgical procedure of intrathecal catheter implantation. The animals in this group were not treated with orexin-A or its vehicle (Saline). 3) Diabetes group. The animals in this group received STZ for induction of diabetes. 4) Diabetes + saline group. The STZ treated (diabetic) animals in this group intrathecally received 10 µL saline (as orexin-A vehicle). 5, 6 and 7) Diabetes + orexin-A treated group. The STZ treated (diabetic) animals in this group intrathecally received orexin-A at dose of 0.6, 2.5 and 5 nM/L (21, 89 and 178 picograms, respectively) (in 10 µL of saline).

2.4. Experimental protocol

Diabetes was induced by a single intraperitoneal injection of 55 mg/kg (i.p.) streptozotocin (STZ) freshly dissolved in 0.1 moL/L citrate buffer (Saleh et al., 2013; Ghosh et al., 2004). The rats which received citrate buffer (as STZ vehicle) were used as control. One week later, diabetes was confirmed in STZ injected rats by measuring serum glucose concentrations (Kaeidi et al., 2013). The serum glucose concentration was assayed enzymatically using a glucose oxidase-peroxidase (GOD-POD) kit (Pars Azmon Co., Iran). The rats with blood glucose ≥250 mg/dL were considered to have diabetes (Rasoulian et al., 2018). One week after the STZ injection, the diabetic rats were given orexin-A (daily, intrathecal). The tail flick test was done every week during the experimental period (weeks 1,2,3,4, and 5). The rotarod treadmill was done before and at the end of the experiment to assess the motor coordination. Five weeks after the STZ injection, the rats were killed by decapitation under CO_2 anesthesia and the lumbar spinal cord of the animals was removed for further molecular evaluations (apoptosis and oxidative stress biomarkers). Control rats were killed in the same way 5 weeks after receiving vehicle injection. The

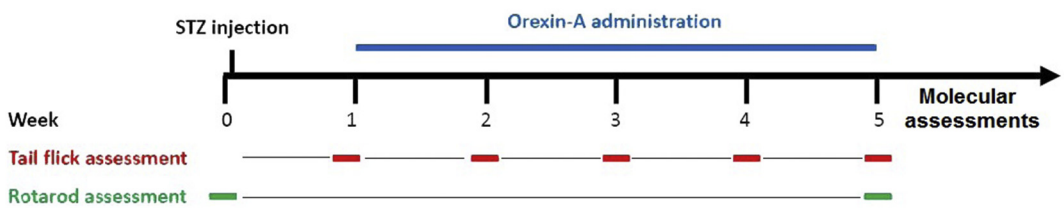

Fig. 1. The experimental protocol design. Animals were divided into 7 groups. On the first day of the experiment period, the STZ was injected. After 1 week, the animals were treated with orexin-A or saline for 5 weeks (week 1 to 5). Tail flick test was done in weeks 1 to 5. Rotarod treadmill was done on day 0 (the day before STZ injection) and on the final day of the experiments. The animals were killed at the end of the experimental period of study and their brains were removed for evaluation of molecular assessments of apoptosis and oxidative stress markers.

experimental procedure is shown in Fig. 1.

2.5. Intrathecal catheter implantation and drug delivery

The rats were deeply anesthetized with co-administration of ketamine (50 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.). An intrathecal catheter (Intramedic PE-10, Becton Dickinson and Co. Franklin Lakes, NJ) was implanted in each rat spinal cord column according to a previously published method (Hajializadeh et al., 2010). Animals that showed neurological impairment (e.g., paralysis) following the implantation of the catheter or during the first drug delivery were excluded from the study. Orexin-A or saline were slowly (10 μL/3 min) injected into the implanted intrathecal catheter.

2.6. Nociception assay

The nociceptive threshold of rats was evaluated by tail-flick test (D'amour and Smith, 1941). This test has been extensively used to evaluate the nociception in rats and mice and is the nociceptive test most frequently used in animals (Le Bars et al., 2001). In the standard method, radiant heat is focused on the animal tail, and the time it takes until the animal flicks the tail away from the light beam is measured. This tail–flick latency is a measure of the nociceptive sensitivity of the animal. In this test, a spinal nociceptive reflex is measured (Bannon and Malmberg, 2007; Carstens and Douglass, 1995). In this study, a tail-flick apparatus with a radiant heat source was used to assess the analgesic response (Ugo Basile, Italy). The intensity of the light beam was adjusted to produce a mean control reaction time of between 6 and 8 s. The cutoff time was fixed at 15 s in order to prevent any injury to the animal's tail. In this manner, we were able to show potential subtle changes that may occur in basal thermal nociception. The tail-flick latency for any rat was determined 3 times, and the mean of those value was designated the baseline latency value. Experimentally decreased tail-flick latency is indicative of hyperalgesia as a marker of neuropathy (Kamenov et al., 2006; Ilnytska et al., 2006).

2.7. Rotarod treadmill

The rotarod treadmill examination was done to evaluate the motor coordination of the rats (Cartmell et al., 1991). The animals were placed on the rotating rod apparatus for two trials each on day 0 (to evaluate the motor coordination between all experimental groups before STZ injection and drug delivery) and in the end of week 5 of the experiments (to evaluate the motor coordination between all experimental groups after STZ injection and drug delivery). The animals were initially trained to maintain themselves on the rotating rod for > 3 min. Then three trials were performed over 3 min. The mean latency to fall off the rotarod was recorded and animals remaining on the drum for > 120 s were removed and their time scored as 120 s. In each trial, the rats were scored for their latency to fall (in seconds).

2.8. Spinal cord tissue extraction and preparation

At the end of the experimental period, the animals were anesthetized by exposure to a CO_2 atmosphere and then rapidly decapitated. The spinal column of animals was cut through the pelvic girdle with sharp stainless steel scissors. Hydraulic extrusion was applied via inserting a 16-gauge plastic needle into the sacral vertebral canal and emitted with ice-cold saline. The spinal cord was rapidly placed on glass petri dish filled with ice-cold saline, then the dorsal half of the spinal cord lumbar portion was dissected (Hajializadeh et al., 2014; Kaeidi et al., 2013; Kaeidi et al., 2011). Tissue samples were rapidly frozen in liquid nitrogen, and stored at −80 °C until molecular investigations (Esmaeili-Mahani et al., 2015).

2.9. Immunoblot analysis

The dissected spinal samples were homogenized in ice-cold RIPA buffer including 1 mM EDTA, 10 mM Tris–HCl (pH 7.4), 0.1% SDS, 0.1% Na deoxycholate, 1% triton X100 with protease inhibitors (2.5 μg/mL of leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 10 μg/mL of aprotinin). The sample homogenates were centrifuged at 14000 rpm for 20 min (4 °C). The subsequent supernatant was reserved as the whole cell fraction. Concentrations of total protein were evaluating via the Bradford method (Bio-Rad, Germany). The same protein amount was resolved electrophoretically on a 12.5% SDS-PAGE gel and subsequently transferred to PVDF membranes. After blocking with 5% non-fat dried milk in Tris-buffered saline with Tween 20 (blocking buffer, TBS-T, 150 mM NaCl, 20 mM Tris–HCl, pH 7.5, 0.1% Tween 20, overnight at 4 °C), the PVDF membranes were probed with caspase-3 antibody (1:5000 overnight at 4 °C), Bax and Bcl-2 antibodies (1:5000 for 2 h at room temperature). After washing in TBS-T (three times, 5 min), the blots were incubated for 60 min at room temperature with a horseradish peroxidase-conjugated secondary antibody (1:5000). All antibodies were diluted in 5% blocking buffer. The antibody-antigen complexes were detected using the ECL system and exposed to Lumi-Film chemiluminescent detection film. The intensity of band expression was evaluated using ImageJ analyzing software. Beta-Actin immunoblotting, (1:5000) was used as a loading control (Rasoulian et al., 2014).

3. Statistical analysis

The data are presented as the mean ± SEM. The difference in mean tail-flick latency (time, seconds) between various experimental groups over the time course of the study (5 weeks) was assessed by repeated measures two-way ANOVA followed by the Tukey post hoc test. The result from the rotarod test was evaluated using one-way ANOVA followed by the Tukey post hoc test. The cleaved caspase-3, Bax, Bcl-2, and beta-actin band densities were taken from band densitometry. These values were expressed as the tested protein/beta-actin ratio for any sample. The averages for significant different groups were compared by one-way ANOVA followed by the Tukey post hoc test. $P^{<}0.05$ was considered significant.

4. Results

4.1. The effect of orexin-A on the nociceptive threshold

As illustrated in Fig. 2, diabetic rats exhibited a hyperalgesic response during the experiment period. The hyperalgesia appeared two weeks after injection of STZ and persisted to the end of investigation (week 5). Intrathecal administration of saline (as orexin-A vehicle) did not show any significant effect on the progress of the mentioned pronociceptive response in diabetic animals. Though 2.5 and 5 nM/L orexin-A could reduce the diabetes-induced hyperalgesia (Fig. 2). Additionally, the higher concentration of orexin-A used here (5 nM/L) produced a reduction of diabetes-induced hyperalgesia, that is, the nociceptive threshold detected in diabetic rats treated with orexin-A was indistinguishable from control or sham rats.. In contrast, treatment with 0.6 nM/L orexin-A did not inhibit the development of diabetes-induced hyperalgesia in rats (Fig. 2).

4.2. The effect of orexin-A on diabetes-induced motor deficits

The rotarod treadmill examination showed a significant deficit of the motor coordination in diabetes and diabetes + saline rats ($P^{<}0.001$) (Fig. 3). The retention time of the diabetic rats was reduced by 58.58% of control animals, while treatment with 5 nM/L orexin-A elevated retention time to 83.93% of the control values (25.35% more than non-treated diabetic animal group). As showed in Fig. 3, there is

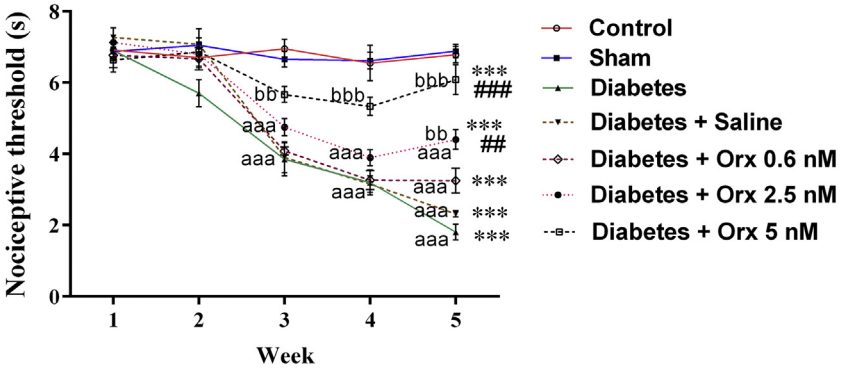

Fig. 2. Effect of orexin-A on the development of diabetes-induced thermal hyperalgesia during the time course of the study in rats. Lines indicate the nociceptive threshold of each experimental group. Values are shown as the mean ± SEM (n = 6–8). **P<0.01 and ***P<0.001 versus control or sham groups (for overall row comparison between the experimental groups during the 5 weeks). ##P<0.01 and ###P<0.001 versus diabetes or diabetes + saline groups (for overall row comparison between the experimental groups during the 5 weeks). aaa P<0.001 versus control or sham (for comparison between the experimental groups in each week). bb P<0.01 and bbb P<0.001 versus diabetes or diabetes + saline groups (for comparison between the experimental groups in each week).

no significant decrease of motor coordination deficit in diabetes +5 nM/L orexin-A treated group compared to control or sham groups (P > .05). Orexin-A at doses of 0.6 and 2.5 nM/L had no significant effects on diabetes-induced motor deficits.

4.3. The effect of orexin-A on apoptotic parameters

We evaluated the orexin-A on some apoptotic molecular factors such as cleaves caspase-3, Bax (as pro-apoptotic protein factors) and Bcl2 (as anti-apoptotic protein factor) by western blotting analysis. Our data in western blotting analysis revealed that cleaved caspase-3 expression was significantly elevated in the dorsal portion of the lumbar spinal cord of diabetic and diabetic + saline rats (P<0.01). The data showed that cleavage of caspase-3 protein was significantly decreased in diabetic rats that were treated with 5 nM/L orexin-A compared to non-treated orexin-A diabetic animals (P<0.05). Orexin-A treatment with other doses could not reduce the cleaved caspase-3 activation in diabetic animals (Fig. 4A). Also, an elevation of Bax and Bcl2 protein expression levels was seen in diabetic animals (P<0.001 and P<0.01 respectively) (Fig. 4B and C). Furthermore, our finding revealed that treatment of diabetic animals with 5 nM/L orexin-A lead to decreases in Bax protein expression versus control or sham groups (P<0.05) (Fig. 4B). On the other hand, the data showed that anti-apoptotic Bcl2 protein expression increased in 5 nM/L orexin-A treated rats versus diabetes or diabetes + saline groups. However, no significant change between control and diabetes +5 nM/L orexin-A treated rats was seen. Treatment of diabetic rats with 0.6 and 2.5 nM/L orexin-A could not elevate the Bax or Bcl2 protein expression (Fig. 4B and C). So, there was a significant increase in the ratio of Bax/Bcl-2 protein in non-treated diabetic rats (P<0.001) (Fig. 4D). This increase in the ratio of Bax/Bcl-2 was attenuated significantly in diabetic rats treated by 5 nM/L orexin-A versus diabetes and diabetes + saline groups (P<0.05) (Fig. 4D). Administration of orexin-A at doses of 0.6 and 2.5 nM/L orexin-A had no significant effects on Bax/Bcl2 ratio expression in diabetic animals compared to control or sham rats (Fig. 4D).

5. Discussion

In the present investigation, the possible protective effect of orexin-A in a rat model of painful diabetic neuropathy was studied. The results showed that non-treated diabetes (which was induced by STZ injection), led to hyperalgesia, motor coordination deficits and apoptosis in the lumbar spinal cord of diabetic animals at the end of study. Furthermore, we find that intrathecal 4-week administration of orexin-A, significantly reduced the thermal-hyperalgesia, motor coordination deficits, and down-regulates some apoptotic protein markers in the diabetes-induced neuronal apoptosis pathway, suggesting that orexin-A may have antihyperalgesic and neuroprotective properties.

Our finding showed that the threshold of nociception was decreased after two weeks at the start of study in diabetic animals. This decrease in nociception threshold persisted until the end of the time course of study (week 5) by incremental manner in each week. This phenomenon may be related to neuronal damage which was triggered and progressed in sensory fibers by hyperglycemia in diabetic animals. On the other hand, treatment with orexin-A attenuated the pain threshold in diabetic animals. The first potential antinocicetion effect of orexin-A was seen at week three and progressed until the end of experimental period in diabetic rats.

As mentioned previously, neuropathic pain (as one of the most common complications of diabetes mellitus) is typically considered as pain that can happen instinctively as a result of experience with mild painful stimuli (Spallone and Greco, 2013). (Peltier et al., 2014). Indeed, the damage and apoptosis in the nerve tissue lead to diabetic neuropathic pain in several studies (Afrazi et al., 2014; Hajializadeh et al., 2014; Kaeidi et al., 2013; Rasoulian et al., 2018).

In the present study, we found orexin-A treatment reduces apoptosis in the spinal cord after diabetic neuropathy induction by modulation of the Bax/Bcl-2 proteins ratio and attenuated caspase-3 activation.

Previously, the loss or dysfunction of orexin neurons has been reported in some neurodegenerative diseases such as multiple sclerosis (Oka et al., 2004), Huntington disease (Gabery et al., 2010), Alzheimer's (Fronczek et al., 2011) and Parkinson's disease (Fronczek et al.,

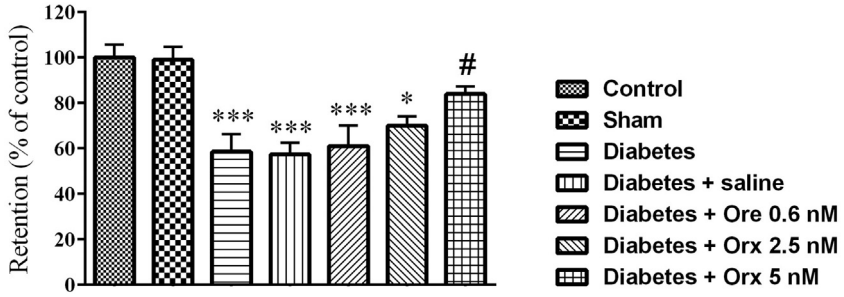

Fig. 3. Effect of orexin-A on the rotarod treadmill performance of streptozotocin-induced diabetic rats. Values are shown as the mean ± SEM (n = 6–8). *P<0.05 and ***P<0.001 versus control or sham groups. #P<0.05 versus diabetes or diabetes + saline groups.

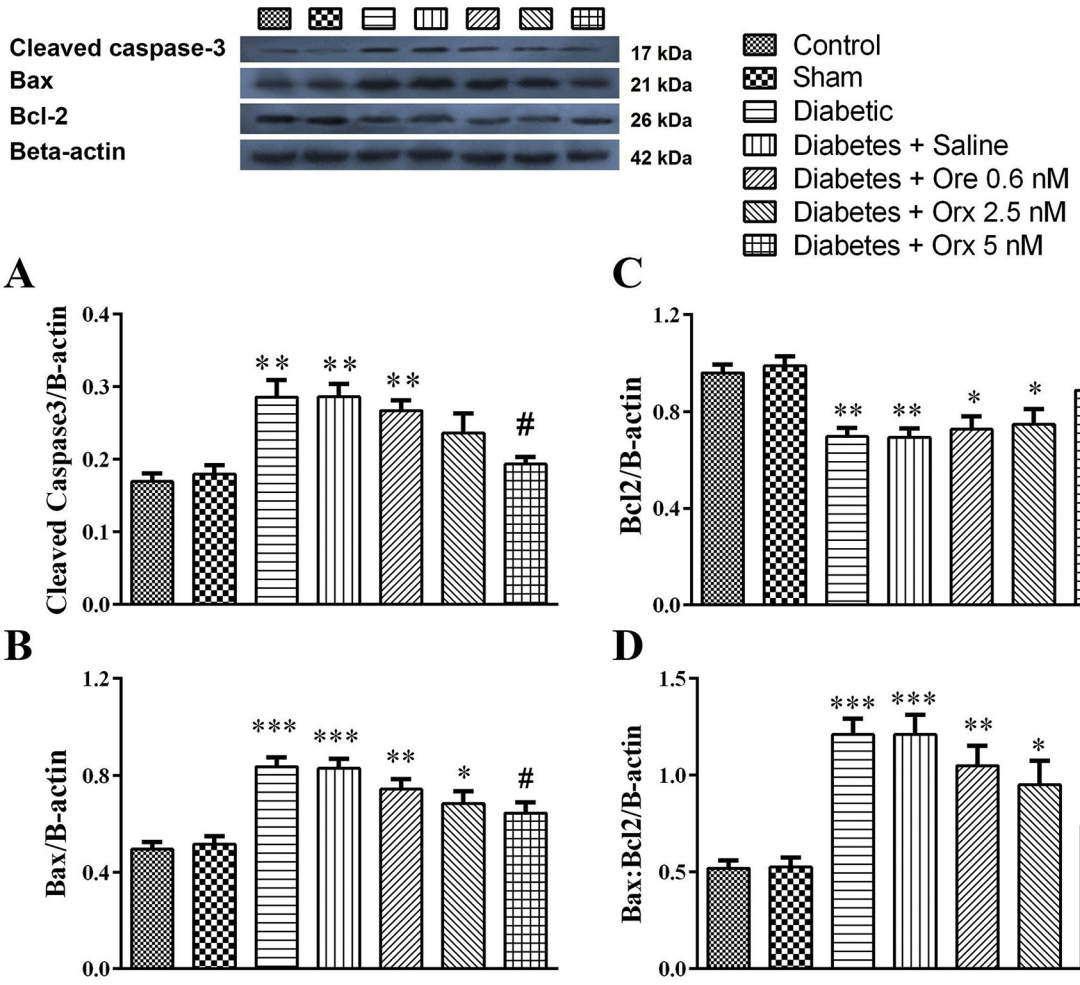

Fig. 4. Western blot analysis of the caspase-3 protein activation (A), Bax (B), Bcl-2 (C), and Bax/Bcl-2 ratio (D) in the dorsal portion of the animal lumbar spinal cord. Each value in the graph represents the mean ± SEM band density ratio for each group. Beta-actin was used as an internal control. *P<0.05, **P<0.01 and ***P<0.001 versus control or sham groups. #P<0.05 versus diabetes or diabetes + saline groups. Orx: orexin-A.

2007b; Fronczek et al., 2007a). Yuan and colleagues reported that orexin-A has a neuroprotective effect against cerebral ischemia-reperfusion injury in rats (Yuan et al., 2011). It has been revealed that central administration of orexin-A significantly reduces the brain infarct area of rats subjected to middle cerebral artery occlusion-reperfusion brain injury model and produces a profound neuroprotective effect (Kitamura et al., 2010). In addition, it has been reported that orexin-A has a protective effect against 6-OHDA-induced SH-SY5Y cell apoptosis by decreasing the caspase-3 activation and Bax/Bcl2 ratio (Esmaeili-Mahani et al., 2013).

In several investigations it has been shown that free radical generation and oxidative stress play critical roles in the development of neuronal apoptosis and neuropathy in diabetes (Brownlee, 2001). It has been revealed that high glucose concentration produces oxidative stress by the auto-oxidation of glucose, which leads to the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) mediators. These mediators cause protein nitrosylation, lipid peroxidation, DNA damage, and apoptosis and have direct toxic effects on nerve tissue (Vincent et al., 2005).

Many studies indicate that various antioxidants compounds have neuroprotective effects in the neurotoxic situation and neurodegenerative disorders (Ghosh et al., 2004; Hajializadeh et al., 2010; Saleh et al., 2013).

It has been shown that orexin-A has suppressive effects against 6-OHDA-induced intracellular ROS production (Esmaeili-Mahani et al., 2013). Furthermore, the antioxidant activity of orexin-A has also been reported in hydrogen peroxide-induced lipid peroxidative stress in hypothalamic cells (Butterick et al., 2012). A recent study has shown that orexin-A exhibited a gastroprotective effect against ischemia-reperfusion-induced damage by decreasing neutrophil activation and lipid peroxidation (Yan et al., 2008).

It is well known that reactive oxygen species can trigger the activation of caspases cascade in apoptosis mechanism (Simon et al., 2000; Kim et al., 2008). Since caspase-3 plays a central role in apoptosis and is supposed to be the final executor of apoptosis pathway, we evaluated the effect of orexin-A on caspase-3 activation in the half lumbar portion of diabetic rats with painful diabetic neuropathy. Numerous scientific reports have demonstrated that neuronal death in hyperglycemia and painful diabetic neuropathy is markedly associated with activation of caspase-3 (Rasoulian et al., 2018; Kaeidi et al., 2011; Joseph and Levine, 2004).

Furthermore, it has been reported that orexin-A receptors in the spinal cord are involved in the modulation of pain and nociceptive transmission in diabetic neuropathy (Cartmell et al., 1991; D'amour and Smith, 1941). Additionally, some investigations indicated that chronic administration of orexin-A can improve some neurological disorders in experimental autoimmune encephalomyelitis (Fatemi et al., 2016), and in a rat model of Parkinson's disease (Hadadianpour et al., 2017).

In this investigation, we find that orexin-A administration can improve the motor coordination defects in diabetic rats. It has been demonstrated that chronic hyperglycemia can lead to motor coordination injury in diabetic animals (Esmaeili Mahani and Kaeidi, 2012;

Hajializadeh et al., 2014; Kaeidi et al., 2013). Furthermore, motor function deficits in human subject with diabetic neuropathy was shown in several studies (Gutierrez et al., 2001; Said et al., 2008). Dysfunction of motor function in diabetes may be related to motor neuron injury in hyperglycemia situation. As mentioned previously, chronic hyperglycemia leads to oxidative stress and apoptosis in the peripheral nervous system (Edwards et al., 2008). So, orexin-A by preventing the oxidative stress and neuronal apoptosis can insert their neuroprotection effects on motor neurons and therefore improve the motor coordination in diabetic animals.

Altogether, the analgesic effects of orexin-A might be mediated through the attenuation of neuronal apoptosis in rats with painful diabetic neuropathy. On the other hand, we suggest that this finding be evaluated by co-administration of the orexin receptor-A and B antagonist (as the main limitation of our study) in further investigations.

6. Conclusion

In conclusion, the results of this study showed that the orexin-A has antinociceptive and neuroprotective effects, as confirmed by behavioral and molecular assessments in a rat model of painful diabetic neuropathy. The orexin-A treatment had a significant effect on the reduction of thermal hyperalgesia and neuronal apoptosis in the spinal cord lumbar portion of the STZ-induced diabetic animals.

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Conflict of interest

There are no conflicts of interest to declare.

References

Afrazi, S., Esmaeili-Mahani, S., Sheibani, V., Abbasnejad, M., 2014. Neurosteroid allopregnanolone attenuates high glucose-induced apoptosis and prevents experimental diabetic neuropathic pain: in vitro and in vivo studies. J. Steroid Biochem. Mol. Biol. 139, 98–103.

Ahmadi-Soleimani, S.M., Ghaemi-Jandabi, M., Azizi, H., Semnanian, S., 2014. Orexin type 1 receptor antagonism in Lateral Paragigantocellularis nucleus attenuates naloxone precipitated morphine withdrawal symptoms in rats. Neurosci. Lett. 558, 62–66.

Ahmadi-Soleimani, S.M., Azizi, H., Gompf, H.S., Semnanian, S., 2017. Role of orexin type-1 receptors in paragiganto-coerulear modulation of opioid withdrawal and tolerance: a site specific focus. Neuropharmacology 126, 25–37.

Bannon, A.W., Malmberg, A.B., 2007. Models of Nociception: Hot-Plate, Tail-Flick, and Formalin Tests in Rodents. (*Current protocols in neuroscience,* 41, 8.9. 1-8.9. 16).

Bingham, S., Davey, P., Babbs, A., Irving, E., Sammons, M., Wyles, M., Jeffrey, P., Cutler, L., Riba, I., Johns, A., 2001. Orexin-a, an hypothalamic peptide with analgesic properties. Pain 92, 81–90.

Boss, C., Roch, C., 2015. Recent trends in orexin research—2010 to 2015. Bioorg. Med. Chem. Lett. 25, 2875–2887.

Brownlee, M., 2001. Biochemistry and molecular cell biology of diabetic complications. Nature 414, 813.

Butterick, T.A., Nixon, J.P., Billington, C.J., Kotz, C.M., 2012. Orexin a decreases lipid peroxidation and apoptosis in a novel hypothalamic cell model. Neurosci. Lett. 524, 30–34.

Carstens, E., Douglass, D., 1995. Midbrain suppression of limb withdrawal and tail flick reflexes in the rat: correlates with descending inhibition of sacral spinal neurons. J. Neurophysiol. 73, 2179–2194.

Cartmell, S.M., Gelgor, L., Mitchell, D., 1991. A revised rotarod procedure for measuring the effect of antinociceptive drugs on motor function in the rat. Journal of pharmacological methods 26, 149–159.

Chrobok, L., Palus-Chramiec, K., Chrzanowska, A., Kepczynski, M., Lewandowski, M.H., 2017. Multiple excitatory actions of orexins upon thalamo-cortical neurons in dorsal lateral geniculate nucleus-implications for vision modulation by arousal. Sci. Rep. 7, 7713.

Coborn, J., Deporter, D., Mavanji, V., Sinton, C., Kotz, C., Billington, C., Teske, J., 2017. Role of orexin-a in the ventrolateral preoptic area on components of total energy expenditure. Int. J. Obes. 41, 1256.

D'amour, F.E., Smith, D.L., 1941. A method for determining loss of pain sensation. J. Pharmacol. Exp. Ther. 72, 74–79.

Date, Y., Mondal, M.S., Matsukura, S., Nakazato, M., 2000. Distribution of orexin-a and orexin-B (hypocretins) in the rat spinal cord. Neurosci. Lett. 288, 87–90.

Davies, J., Chen, J., Pink, R., Carter, D., Saunders, N., Sotiriadis, G., Bai, B., Pan, Y., Howlett, D., Payne, A., 2015. Orexin receptors exert a neuroprotective effect in Alzheimer's disease (AD) via heterodimerization with GPR103. Sci. Rep. 5, 12584.

Duffy, C.M., Nixon, J.P., Butterick, T.A., 2016. Orexin a attenuates palmitic acid-induced hypothalamic cell death. Mol. Cell. Neurosci. 75, 93–100.

Edwards, J.L., Vincent, A.M., Cheng, H.T., Feldman, E.L., 2008. Diabetic neuropathy: mechanisms to management. Pharmacol. Ther. 120, 1–34.

Esmaeili Mahani, S., Kaeidi, A., 2012. Olive (Olea europaea L.) leaf extract prevents motor deficit in streptozotocin-induced diabetic rats. Physiology and Pharmacology 16, 156–164.

Esmaeili-Mahani, S., Vazifekhah, S., Pasban-Aliabadi, H., Abbasnejad, M., Sheibani, V., 2013. Protective effect of orexin-a on 6-hydroxydopamine-induced neurotoxicity in SH-SY5Y human dopaminergic neuroblastoma cells. Neurochem. Int. 63, 719–725.

Esmaeili-Mahani, S., Ebrahimi, B., Abbasnejad, M., Rasoulian, B., Sheibani, V., 2015. Satureja khuzestanica prevents the development of morphine analgesic tolerance through suppression of spinal glial cell activation in rats. J. Nat. Med. 69, 165–170.

Fatemi, I., Shamsizadeh, A., Ayoobi, F., Taghipour, Z., Sanati, M.H., Roohbakhsh, A., Motevalian, M., 2016. Role of orexin-a in experimental autoimmune encephalomyelitis. J. Neuroimmunol. 291, 101–109.

Feng, Y., Liu, T., Li, X.-Q., Liu, Y., Zhu, X.-Y., Jankovic, J., Pan, T.-H., Wu, Y.-C., 2014. Neuroprotection by Orexin-a via HIF-1α induction in a cellular model of Parkinson's disease. Neurosci. Lett. 579, 35–40.

Fronczek, R., Overeem, S., Lee, S.Y., Hegeman, I.M., Van Pelt, J., Van Duinen, S.G., Lammers, G.J., Swaab, D.F., 2007a. Hypocretin (orexin) loss and sleep disturbances in Parkinson's Disease. Brain 131, e88.

Fronczek, R., Overeem, S., Lee, S.Y., Hegeman, I.M., Van Pelt, J., Van Duinen, S.G., Lammers, G.J., Swaab, D.F., 2007b. Hypocretin (orexin) loss in Parkinson's disease. Brain 130, 1577–1585.

Fronczek, R., Van Geest, S., Frlich, M., Overeem, S., Roelandse, F.W., Lammers, G., Swaab, D., 2011. Hypocretin (orexin) loss in Alzheimer's disease. Sleep & Biological Rhythms 9, 328.

Gabery, S., Murphy, K., Schultz, K., Loy, C.T., Mccusker, E., Kirik, D., Halliday, G., Petersén, Å., 2010. Changes in key hypothalamic neuropeptide populations in Huntington disease revealed by neuropathological analyses. Acta Neuropathol. 120, 777–788.

Ghaemi-Jandabi, M., Azizi, H., Ahmadi-Soleimani, S.M., Semnanian, S., 2017. Intracoerulear microinjection of orexin-a induces morphine withdrawal-like signs in rats. Brain Res. Bull. 130, 107–111.

Ghosh, S., Qi, D., An, D., Pulinilkunnil, T., Abrahani, A., Kuo, K.-H., Wambolt, R.B., Allard, M., Innis, S.M., Rodrigues, B., 2004. Brief episode of STZ-induced hyperglycemia produces cardiac abnormalities in rats fed a diet rich in n-6 PUFA. Am. J. Phys. Heart Circ. Phys. 287, H2518–H2527.

Grudt, T.J., Van Den Pol, A.N., Perl, E.R., 2002. Hypocretin-2 (orexin-B) modulation of superficial dorsal horn activity in rat. J. Physiol. 538, 517–525.

Gutierrez, E.M., Helber, M.D., Dealva, D., Ashton-Miller, J.A., Richardson, J.K., 2001. Mild diabetic neuropathy affects ankle motor function. Clin. Biomech. 16, 522–528.

Hadadianpour, Z., Fatehi, F., Ayoobi, F., Kaeidi, A., Shamsizadeh, A., Fatemi, I., 2017. The effect of orexin-a on motor and cognitive functions in a rat model of Parkinson's disease. Neurol. Res. 39, 845–851.

Hajializadeh, Z., Esmaeili-Mahani, S., Sheibani, V., Kaeidi, A., Atapour, M., Abbasnejad, M., 2010. Changes in the gene expression of specific G-protein subunits correlate with morphine insensitivity in streptozotocin-induced diabetic rats. Neuropeptides 44, 299–304.

Hajializadeh, Z., Nasri, S., Kaeidi, A., Sheibani, V., Rasoulian, B., Esmaeili-Mahani, S., 2014. Inhibitory effect of Thymus caramanicus Jalas on hyperglycemia-induced apoptosis in in vitro and in vivo models of diabetic neuropathic pain. J. Ethnopharmacol. 153, 596–603.

Hervieu, G., Cluderay, J., Harrison, D., Roberts, J., Leslie, R., 2001. Gene expression and protein distribution of the orexin-1 receptor in the rat brain and spinal cord. Neuroscience 103, 777–797.

Ilnytska, O., Lyzogubov, V.V., Stevens, M.J., Drel, V.R., Mashtalir, N., Pacher, P., Yorek, M.A., Obrosova, I.G., 2006. Poly (ADP-ribose) polymerase inhibition alleviates experimental diabetic sensory neuropathy. Diabetes 55, 1686–1694.

Joseph, E.K., Levine, J.D., 2004. Caspase signalling in neuropathic and inflammatory pain in the rat. Eur. J. Neurosci. 20, 2896–2902.

Kaeidi, A., Esmaeili-Mahani, S., Sheibani, V., Abbasnejad, M., Rasoulian, B., Hajializadeh, Z., Afrazi, S., 2011. Olive (Olea europaea L.) leaf extract attenuates early diabetic neuropathic pain through prevention of high glucose-induced apoptosis: in vitro and in vivo studies. J. Ethnopharmacol. 136, 188–196.

Kaeidi, A., Esmaeili-Mahani, S., Abbasnejad, M., Sheibani, V., Rasoulian, B., Hajializadeh, Z., Pasban-Aliabadi, H., 2013. Satureja khuzestanica attenuates apoptosis in hyperglycemic PC12 cells and spinal cord of diabetic rats. J. Nat. Med. 67, 61–69.

Kaeidi, A., Taati, M., Hajializadeh, Z., Jahandari, F., Rashidipour, M., 2015. Aqueous extract of Zizyphus jujuba fruit attenuates glucose induced neurotoxicity in an in vitro model of diabetic neuropathy. Iranian journal of basic medical sciences 18, 301.

Kamenov, Z., Higashino, H., Todorova, M., Kajimoto, N., Suzuki, A., 2006. Physiological characteristics of diabetic neuropathy in sucrose-fed otsuka Long-Evans Tokushima fatty rats. Methods Find. Exp. Clin. Pharmacol. 28, 13–18.

Kargar, H.M.-P., Azizi, H., Mirnajafi-Zadeh, J., Reza, M.A., Semnanian, S., 2015. Microinjection of orexin-a into the rat locus coeruleus nucleus induces analgesia via cannabinoid type-1 receptors. Brain Res. 1624, 424–432.

Kim, J.S., Lee, J.H., Jeong, W.W., Choi, D.H., Cha, H.J., Kim, D.H., Kwon, J.K., Park, S.E., Park, J.H., Cho, H.R., 2008. Reactive oxygen species-dependent EndoG release mediates cisplatin-induced caspase-independent apoptosis in human head and neck squamous carcinoma cells. Int. J. Cancer 122, 672–680.

Kitamura, E., Hamada, J., Kanazawa, N., Yonekura, J., Masuda, R., Sakai, F., Mochizuki, H., 2010. The effect of orexin-a on the pathological mechanism in the rat focal cerebral ischemia. Neurosci. Res. 68, 154–157.
Kukkonen, J.P., 2012. Recent progress in orexin/hypocretin physiology and pharmacology. Biomolecular concepts 3, 447–463.
Le Bars, D., Gozariu, M., Cadden, S.W., 2001. Animal models of nociception. Pharmacol. Rev. 53, 597–652.
Lee, H.-J., Chang, L.-Y., Ho, Y.-C., Teng, S.-F., Hwang, L.-L., Mackie, K., Chiou, L.-C., 2016. Stress induces analgesia via orexin 1 receptor-initiated endocannabinoid/CB1 signaling in the mouse periaqueductal gray. Neuropharmacology 105, 577–586.
Lu, X.-Y., Bagnol, D., Burke, S., Akil, H., Watson, S.J., 2000. Differential distribution and regulation of OX1 and OX2 orexin/hypocretin receptor messenger RNA in the brain upon fasting. Horm. Behav. 37, 335–344.
Mobarakeh, J.I., Takahashi, K., Sakurada, S., Nishino, S., Watanabe, H., Kato, M., Yanai, K., 2005. Enhanced antinociception by intracerebroventricularly and intrathecally-administered orexin a and B (hypocretin-1 and-2) in mice. Peptides 26, 767–777.
Oka, Y., Kanbayashi, T., Mezaki, T., Iseki, K., Matsubayashi, J., Murakami, G., Matsui, M., Shimizu, T., Shibasaki, H., 2004. Low CSF hypocretin-1/orexin-a associated with hypersomnia secondary to hypothalamic lesion in a case of multiple sclerosis. J. Neurol. 251, 885–886.
Ossipov, M.H., Dussor, G.O., Porreca, F., 2010. Central modulation of pain. J. Clin. Invest. 120, 3779–3787.
Peltier, A., Goutman, S.A., Callaghan, B.C., 2014. Painful diabetic neuropathy. BMJ: British Medical Journal (Online) 348.
Peyron, C., Tighe, D.K., Van Den Pol, A.N., De Lecea, L., Heller, H.C., Sutcliffe, J.G., Kilduff, T.S., 1998. Neurons containing hypocretin (orexin) project to multiple neuronal systems. J. Neurosci. 18, 9996–10015.
Rasoulian, B., Kaeidi, A., Pourkhodadad, S., Dezfoulian, O., Rezaei, M., Wahhabaghai, H., Alirezaei, M., 2014. Effects of pretreatment with single-dose or intermittent oxygen on Cisplatin-induced nephrotoxicity in rats. Nephro-urology monthly 6.
Rasoulian, B., Hajializadeh, Z., Esmaeili-Mahani, S., Rashidipour, M., Fatemi, I., Kaeidi, A., 2018. Neuroprotective and antinociceptive effects of rosemary (Rosmarinus officinalis L.) extract in rats with painful diabetic neuropathy. J. Physiol. Sci. 1–8.
Razavi, B.M., Hosseinzadeh, H., 2017. A review of the role of orexin system in pain modulation. Biomed. Pharmacother. 90, 187–193.
Saberi Firouzi, S., Namazi Sarvestani, N., Bakhtiarian, A., Ghazi Khansari, M., Karimi, M.Y., Ranjbar, A., Safa, M., Hosseini, A., 2018. Sildenafil protective effects on high glucose-induced neurotoxicity in PC12 cells: the role of oxidative stress, apoptosis, and inflammation pathways in an in vitro cellular model for diabetic neuropathy. Neurol. Res. 1–13.
Said, G., Baudoin, D., Toyooka, K., 2008. Sensory loss, pains, motor deficit and axonal regeneration in length-dependent diabetic polyneuropathy. J. Neurol. 255, 1693.
Saleh, D.O., Bayoumi, A.R., El-Eraky, W.I., El-Khatib, A.S., 2013. Streptozotocin-induced vascular and biochemical changes in rats: Effects of rosiglitazone vs. metformin. Bulletin of Faculty of Pharmacy, Cairo University 51, 131–138.
Sekiguchi, M., Sekiguchi, Y., Konno, S.-I., Kobayashi, H., Homma, Y., Kikuchi, S.-I., 2009. Comparison of neuropathic pain and neuronal apoptosis following nerve root or spinal nerve compression. Eur. Spine J. 18, 1978.
Shu, Q., Zhang, J., Ma, W., Lei, Y., Zhou, D., 2017. Orexin-a promotes Glu uptake by OX1R/PKCα/ERK1/2/GLT-1 pathway in astrocytes and protects co-cultured astrocytes and neurons against apoptosis in anoxia/hypoglycemic injury in vitro. Mol. Cell. Biochem. 425, 103–112.
Simon, H.-U., Haj-Yehia, A., Levi-Schaffer, F., 2000. Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis 5, 415–418.
Soleimani, S.M.A., Azizi, H., Mirnajafi-Zadeh, J., Semnanian, S., 2015. Orexin type 1 receptor antagonism in rat locus coeruleus prevents the analgesic effect of intra-LC met-enkephalin microinjection. Pharmacol. Biochem. Behav. 136, 102–106.
Spallone, V., Greco, C., 2013. Painful and painless diabetic neuropathy: one disease or two? Current diabetes reports 13, 533–549.
Toyama, S., Shimoyama, N., Shimoyama, M., 2017. The analgesic effect of orexin-a in a murine model of chemotherapy-induced neuropathic pain. Neuropeptides 61, 95–100.
Trivedi, P., Yu, H., MacNeil, D.J., Van Der Ploeg, L., Guan, X.-M., 1998. Distribution of orexin receptor mRNA in the rat brain. FEBS Lett. 438, 71–75.
Van Den Pol, A.N., 1999. Hypothalamic hypocretin (orexin): robust innervation of the spinal cord. J. Neurosci. 19, 3171–3182.
Vincent, A.M., Mclean, L.L., Backus, C., Feldman, E.L., 2005. Short-term hyperglycemia produces oxidative damage and apoptosis in neurons. FASEB J. 19, 638–640.
Wang, C.-M., Yang, C.-Q., Cheng, B.-H., Chen, J., Bai, B., 2018. Orexin-A protects SH-SY5Y cells against H2O2-induced oxidative damage via the PI3K/MEK1/2/ERK1/2 signaling pathway. Int. J. Immunopathol. Pharmacol. 32 (2058738418785739).
Yamada, H., Okumura, T., Motomura, W., Kobayashi, Y., Kohgo, Y., 2000. Inhibition of food intake by central injection of anti-orexin antibody in fasted rats. Biochem. Biophys. Res. Commun. 267, 527–531.
Yan, J., Ge, L., Huang, W., Song, B., Chen, X., Yu, Z., 2008. Orexin affects dorsal root ganglion neurons: a mechanism for regulating the spinal nociceptive processing. Physiol. Res. 57, 797.
Yang, S., Xia, C., Li, S., Du, L., Zhang, L., Hu, Y., 2014. Mitochondrial dysfunction driven by the LRRK2-mediated pathway is associated with loss of Purkinje cells and motor coordination deficits in diabetic rat model. Cell Death Dis. 5, e1217.
Yuan, L.-B., Dong, H.-L., Zhang, H.-P., Zhao, R.-N., Gong, G., Chen, X.-M., Zhang, L.-N., Xiong, L., 2011. Neuroprotective effect of orexin-a is mediated by an increase of hypoxia-inducible factor-1 activity in rat. Anesthesiology: The Journal of the American Society of Anesthesiologists 114, 340–354.
Zimmet, P., Alberti, K.G., Magliano, D.J., Bennett, P.H., 2016. Diabetes mellitus statistics on prevalence and mortality: facts and fallacies. Nat. Rev. Endocrinol. 12, 616.