OX_2 receptors mediate the inhibitory effects of orexin-A on potassium chloride-induced increases in intracellular calcium ion levels in neurons derived from rat dorsal root ganglion in a chronic pain model

Masami Yamaguchi[a], Manabu Ishikawa[a], Yuri Aono[b], Tadashi Saigusa[b]

[a]Department of Anesthesiology, Nihon University School of Dentistry at Matsudo, 2-870-1 Sakaecho-Nishi, Matsudo, Chiba 271-8587, Japan

[b]Department of Pharmacology, Nihon University School of Dentistry at Matsudo, 2-870-1 Sakaecho-Nishi, Matsudo, Chiba 271-8587, Japan

Corresponding author:

Dr. Tadashi Saigusa

Department of Pharmacology, Nihon University School of Dentistry at Matsudo, 2-870-1 Sakaecho-Nishi, Matsudo, Chiba 271-8587, Japan

Tel.: +81-47-360-9344; Fax: +81-47-360-9348

E-mail address: saigusa.tadashi@nihon-u.ac.jp (T. Saigusa)

Abstract

Aims: Orexin-A is known to induce anti-nociceptive effects in animal models of chronic pain. We have found that orexin-A inhibits KCl loading-induced increases in the intracellular calcium ion levels ($[Ca^{2+}]_i$) in C-fiber-like neurons of rats showing inflammatory nociceptive behaviour. Here, we examined the effects of orexin-A on the depolarisation of C-fiber-like neurons derived from a rat model for another type of chronic pain, namely neuropathic pain. Thus, we analysed the effects of orexin-A on KCl-induced increases in $[Ca^{2+}]_i$ in C-fiber-like neurons of rats with sciatic nerve ligation.

Methods: Paw withdrawal and threshold force in response to tactile stimuli were evaluated using von Frey filaments. Sham-operated rats served as controls. $[Ca^{2+}]_i$ in neurons were visualised by calcium fluorescent probe. Changes in $[Ca^{2+}]_i$ were assessed using relative fluorescence intensity.

Results: Seven days after sciatic nerve ligation, paw withdrawal and threshold force for tactile stimuli were increased and reduced, respectively. KCl loading to neurons from either sciatic nerve-ligated or control rats increased relative fluorescence intensity. The KCl-induced increase in relative fluorescence intensity in sciatic nerve-ligated, but not that of control rats, was inhibited by orexin-A. The OX_1 and OX_2 receptor antagonist MK-4305 and OX_2 receptor antagonist EMPA, but not the OX_1 receptor antagonist SB 334867, each counteracted orexin-A-induced inhibition of KCl-provoked increases in relative fluorescence intensity.

Conclusion: The present findings constitute neuropharmacological evidence that OX_2 but not OX_1 receptors mediate the inhibitory effects of orexin-A on KCl-induced increases in $[Ca^{2+}]_i$ in C-fiber-like neurons of rats showing hyper-algesia provoked by sciatic nerve ligation.

Keywords: Orexin receptor; Orexin-A; Intracellular calcium ion levels; Chronic pain; Rat

Abbreviations: $[Ca^{2+}]_i$, intracellular calcium ion levels; KCl, potassium chloride; DRG, dorsal root ganglion; ANOVA, analysis of variance; EDTA, ethylenediaminetetraacetic acid

Introduction

Orexin-A and orexin-B are neuropeptides that are known to be involved in the sleep/wakefulness cycle and in food consumption. These peptides act via two pharmacologically distinct orexin receptor subtypes, namely OX_1 and OX_2 receptors. Both OX_1[1] and OX_2[2] orexin receptor have been found throughout the central nervous system, including the spinal cord. Though orexin-B shows lower affinity for OX_1 receptors than for OX_2 receptors, orexin-A has comparable affinities for both OX_1 and OX_2 receptors.[3]

Orexin-A has been shown to play a stimulatory role in the regulation of feeding[4] and to modulate energy homeostasis.[5] Furthermore, orexin-A is also known to induce anti-nociceptive effects in experimental animal models of chronic pain. Thus, since there are at least two types of chronic pain, called inflammatory nociceptive pain and neuropathic pain, the systemic administration of orexin-A could inhibit inflammatory thermal hyperalgesia in mice induced by intra-planter application of carrageenan without mediating anti-inflammatory processes.[6] Interestingly, intrathecal administration of orexin-A was able to attenuate levels of experimentally-induced mechanical allodynia of rats with sciatic nerve ligations.[7] Similarly, intrathecal administration of orexin-A supressed hyperalgesia in streptozotocin-induced diabetic rats, known to be an animal model of neuropathic pain.[8] These reports strongly suggest that intrathecally-administered orexin-A may alter spinal nociceptive neural transmission. That is, orexin-A may affect the input of primary sensory neurons to the spinal cord. This is because the spinal cord receives afferents of primary sensory neurons, including C-fibers that are excited to transmit peripheral mechanical stimuli.[9] However, the mechanisms of orexin-A's action on excitation of sensory neuronal cells derived from experimental animals under nociceptive stimulation have remained unknown.

We have recently found that potassium chloride (KCl) loading to C-fiber-like small size neurons isolated from dorsal root ganglion (DRG) of rats, either with or without the intraplantar injection of carrageenan to induce inflammatory nociceptive behaviour, increased the intracellular calcium ion levels ($[Ca^{2+}]_i$) of these neurons.[10] We have also found that in the presence of orexin-A, the KCl-provoked increase in $[Ca^{2+}]_i$ of these small size neurons in rats receiving carrageenan injection was supressed.[10] Interestingly, orexin-A did not alter the KCl-provoked increase in $[Ca^{2+}]_i$ of these C-fiber-like small size neurons in rats that did not receive carrageenan injection. These results clearly indicate that orexin-A could inhibit depolarisation of C-fiber-like neural cells in DRG derived from rats with inflammatory nociceptive pain, but not from those without pain. Peripheral nerve damage could induce a series of cellular pathophysiological changes related to hyperalgesia and neuropathic pain. In contrast to inflammatory nociceptive pain, neuropathic pain often shows resistance to the anti-nociceptive actions of opioids[11]. Neuroplastic changes at peripheral, spinal and supraspinal levels appear to contribute to the low efficacy of mu-opioid receptor agonists, a group of opioids used to manage neuropathic pain[11]. Here, we examined the effects of orexin-A on depolarisation of C-fiber-like neural cells derived from a rat model for another type of chronic pain, namely neuropathic pain. Thus, in the present study we analysed the effects of orexin-A on KCl-induced increases in $[Ca^{2+}]_i$ in C-fiber-like neurons of rats with sciatic nerve ligation to experimentally induce neuropathic pain.

First, we carried out a detailed analysis of behavioural changes in the withdrawal of rat hind paws after sciatic nerve ligation. We used von Frey filaments to apply a tactile stimulus to the centre of the plantar surface of hind paws and evaluated withdrawal threshold and withdrawal response once per day for one to two weeks. These tests revealed that, from day two to four through to day seven after

sciatic nerve ligation, rats showed a stable reduction in paw withdrawal threshold and an increase in paw withdrawal response to tactile stimulation of the hind paws.

Accordingly, we next isolated neuronal cells in DRG from rats at seven days following sciatic nerve ligation. Based on our previous experimental procedures,[10] these cells were placed on glass adhesive-coated slips on the bottom of an acrylic analytical chamber. Then, KCl loading-provoked increases in $[Ca^{2+}]_i$ were visualised by confocal laser scanning microscopy using a calcium fluorescent probe. We focused on small size neuronal cells, a size of approximately 20 μm, since these cells are suggested to be C-fibers.[12] Changes in $[Ca^{2+}]_i$ in these small size neurons were evaluated by determining relative fluorescence intensity.

Finally, we investigated the effects of orexin-A in the presence or absence of MK-4305, an antagonist at both OX_1 and OX_2 receptor subtypes, SB 334867, a selective antagonist at the OX_1 receptor subtype, and EMPA, a selective antagonist at the OX_2 receptor subtype, on KCl-induced increases in relative fluorescence intensity in these neurons. The present study revealed that SB 334867 was without effect, while MK-4305 and EMPA counteracted the effects of orexin-A on KCl-induced increase in $[Ca^{2+}]_i$ of neuronal cells derived from rats with sciatic nerve ligation. For comparison, we analysed the effects of EMPA on inhibition by orexin-A of the KCl-induced increase in $[Ca^{2+}]_i$ of neurons derived from rats with inflammation induced by intraplantar injection of carrageenan into the hind paw. This is because we have suggested that OX_2 receptors mediate inhibition by orexin-A of the KCl-induced increase in $[Ca^{2+}]_i$ of C-fiber-like small size neural cells derived from carrageenan-treated rats,[10] but there has been no apparent neuropharmacological evidence to support this proposition.

Materials and methods

Animals

Male Wistar rats (Sankyo Laboratory Service Co. Ltd., Tokyo, Japan), weighing between 225 and 275 g at the start of the experiments, were used. Rats were kept at constant room temperature (23 ± 2 ºC) and relative humidity (55 ± 5%) under a 12 h day:night cycle (light on: 0700 a.m.), with *ad libitum* access to food and water. Their body weight was measured every day and postoperative well-being was routinely monitored. All experiments were approved by the Animal Experimentation Committee of Nihon University School of Dentistry at Matsudo and were performed in accordance with national and international guidelines for the care and welfare of animals. All efforts were made to minimise animal suffering and to reduce the number of animals used.

Sciatic nerve ligation

Sciatic nerve ligation was carried out based on previously described procedures.[13] Briefly, rats were anesthetised with sodium pentobarbital (50 mg/kg i.p.) and placed in the prone position. The paraspinal muscles were separated from the spinous processes at the L2-L4 levels and the L6 transverse processes were carefully removed to identify visually the L4-L6 spinal nerves. The L5 and L6 spinal nerves were isolated and tightly ligated with 3-0 silk thread and the wounds were then sutured. In control rats, the nerve was exposed without ligation.

Rats received bilateral sciatic nerve ligation. This is because experimentally induced inflammation by unilateral injection of carrageenan into a hind paw of rats is well known to enhance nociceptive sensitivity in the contralateral paw.[14] Thus, the untreated side, in which nociceptive perception could be affected by the treated side,

was not suitable as a control in these experiments. Therefore, in order to provide balanced stimulation to the both sides, sciatic nerve was ligated bilaterally (see section *Carrageenan treatment*). Control rats received a sham operation without ligation of the sciatic nerve.

Evaluation of paw withdrawal in response to a tactile stimulus

On the day of behavioural experiments, 1-7 and 14 days after sciatic nerve ligation, rats were placed individually on an elevated nylon mesh floor (50 cm square, 30 cm high) at least 1h before application of the tactile stimulus.

Paw withdrawal in response to a tactile stimulus and threshold forces were assessed using von Frey filaments (DanMic Global, San Jose, CA, USA). Thus, sensitivity to a tactile stimulus was evaluated using von Frey filaments with different bending forces (10 and 26 g). Each von Frey filament was applied to the centre of the plantar surface of the hind paws for 3 s and this was repeated on three occasions per paw. Each of the hind paws of rats was tested individually.

Paw withdrawal behaviour in response to a tactile stimulus was scored as follows: 0, no response; 1, a slow and/or slight response to the stimulus; 2, a quick withdrawal response away from the stimulus without flinching or licking; 3, an intense withdrawal response away from the stimulus with brisk flinching and/or licking. Paw withdrawal in response to each filament was determined as mean scores obtained from the hind paws of each rat. Paw movements associated with locomotion or weight shift were not recorded as a response.

The threshold force (g) that induces a slow and/or slight paw withdrawal to the stimulus was also measured daily immediately before sciatic nerve ligation (day 0); subsequently, one to 14 days after nerve ligation, paw withdrawal threshold was determined as the mean threshold force (g) evaluated for the hind paws of each rat.

Carrageenan treatment

Rats were anaesthetised with sodium pentobarbital (50 mg/kg i.p.) and carrageenan (5 mg lambda carrageenan, Sigma-Aldrich, St. Louis, MO, USA) suspended in 0.25 ml saline was injected subcutaneously into the plantar surface of hind paws via a 27-gauge needle. In control rats, the same volume of saline (0.25 ml) was injected subcutaneously into the plantar surface of hind paws.

For reasons based on our previous studies,[10] as outlined above (see section *Sciatic nerve ligation*), rats here received bilateral injection of carrageenan or vehicle (saline) into the hind paws, with control rats receiving bilateral injection of saline into the hind paws; subsequently, bilateral sciatic nerve ligation was carried out.

Cell culture

Based on paw withdrawal in response to a tactile stimulus, both here and as in a previous study,[10] bilateral sciatic ganglia associated with the sciatic nerve were carefully removed seven days after sciatic nerve ligation and one day after carrageenan treatment. Briefly, DRG were dissected from lumbar vertebrae between L4 and L6 of the spinal column of rats euthanised by carbon dioxide inhalation. Nerve fibers extending from the ganglia were removed under a stereoscopic microscope. Neurons derived from DRG were incubated at 37 ºC in Ca^{2+}-Mg^{2+}-free Hank's balanced salt solution (HBSS (−)) containing 10% fetal bovine serum (FBS, 5 mg/ml, Sigma-Aldrich Co. Ltd., Tokyo, Japan), 5% penicillin (1000 units/ml), streptomycin (10000 μg/ml) and collagenase (3 mg/ml, Collagenase Type 1, Wako Ltd., Osaka, Japan) for 120 min with shaking. These neurons were then centrifuged at 700 rpm for 3 min and rinsed with fresh HBSS (−) in order to remove enzymes.

Subsequently, neurons derived from DRG were incubated at 37 ºC in fresh HBSS (−) containing trypsin (1 mg/l) for 15 min with shaking. Then, the neurons were again centrifuged at 700 rpm for 3 min and rinsed with fresh HBSS (−) in order to further remove enzymes.

Isolated neurons were suspended in Hank's balanced salt solution containing Ca^{2+} and Mg^{2+} (HBSS (＋)). The suspension (1 ml) was collected in an acrylic chamber equipped with glass slips on the bottom coated with an adhesive (Cell-Tak, BD Bioscience, Franklin Lakes, USA) and kept in an incubator for 60 min at 37 ºC. This procedure allowed placement of neurons onto the glass slips for real-time monitoring of neuronal $[Ca^{2+}]_i$ (see section *$[Ca^{2+}]_i$ measurement*).

$[Ca^{2+}]_i$ measurement

The central regions of small-size neurons (approximately 15-25 μm diameter) derived from DRG were selected as regions of interest. Real-time alteration of $[Ca^{2+}]_i$ in these cells was visualised by means of confocal laser scanning microscopy using the calcium fluorescent probe fluo-4-acetoxymethyl ester (Fluo-4/AM, Dojindo laboratories, Kumamoto, Japan). Changes in $[Ca^{2+}]_i$ were assessed as the relative fluorescence intensity (F/F_0 %; F: fluorescence intensity at different time points after KCl treatment; F_0: baseline fluorescence intensity).

Briefly, neurons isolated from DRG and kept in an acrylic chamber (see section *Cell culture*) were incubated at 37 ºC in HBSS (＋) containing Fluo-4/AM (2.2 μM) and 0.1% pluronic F127 for 60 min. Subsequently, supernatants were removed and fresh 37 ºC HBSS (+), used as a bath solution, was added to the acrylic chamber. The chamber was then placed on the stage of a confocal microscope (LSM5Exciter-ZEN 2007, Carl Zeiss Micro Imaging Co. Ltd., Jena, Germany) and fluorescence measurements in these small size neurons were carried out at room

temperature (20-22 °C). In order to minimise any fall in temperature of the bath solution from physiological range (34-37°C), all measurements were done within 10 min. The excitation wavelength of dye in the cytoplasm of selected neuronal cells was 488 nm, with the fluorescent intensity of images determined at 510 nm. Images were captured by a ZEN lite 2011 image analyser (Carl Zeiss Micro Imaging Co. Ltd., Jena, Germany) with an interval of 1 s. These images were further analysed off-line.

Real-time measurement of $[Ca^{2+}]_i$ in an isolated neuron started with determination of basal fluorescent intensity during a period of at least 60 s. Vehicle, orexin-A, orexin-A + SB 334867, orexin-A + MK-4305 or orexin-A + EMPA were added to the bath solution. Then, after 60 s, KCl was added to the bath solution. Changes in fluorescence intensity subsequent to basal fluorescent intensity were recorded for 90 s.

After completion of fluorescence measurements for each neural cell, 1 μM ionomycin, an ionophore, was applied to confirm neuronal viability.

Drugs

Drugs applied to the isolated neurons were: orexin-A (Pyr-Pro-Leu-Pro-Asp-Cys-Cys-Arg-Gln-Lys-Thr-Cys-Ser-Cys-Arg-Leu-Tyr-Glu-Leu-Leu-His-Gly-Ala-Gly-Asn-His-Ala-Ala-Gly-Ile-Leu-Thr-Leu-NH_2, Peptide Institute, Inc., Osaka, Japan); MK-4305 ([(7R)-4-(5-Chloro-1,3-benzoxazol-2-yl)-7-methyl-1,4-diazepan-1-yl][5-methyl-2-(2H-1,2,3-triazol-2-yl)phenyl], ChemScene, LLC, NJ, USA); SB 334867 (N-(2-Methyl-6-benzoxazolyl)-N'-1,5-naphthyridin-4-yl urea, Tocris Bioscience, Ellisville, MO, USA); EMPA (N-Ethyl-2-[(6-methoxy-3-pyridinyl)[(2-methylphenyl)sulfonyl]amino]-N-(3-pyridi

nylmethyl)-acetamide, Sigma-Aldrich, St. Louis, MO, USA); ionomycin (Sigma-Aldrich, St. Louis, MO, USA). These drugs were dissolved in (HBSS (+)) with a small amount of dimethyl sulfoxide (<0.1%) added to the bath solution. Each dose indicates the total amount (nmol) in the 2 ml analytical chamber. Doses of KCl and ionomycin indicate the concentration (mM) in the analytical chamber. Doses used were determined by a series of pilot experiments based on the outcome of previously reported studies.[15-19]

The vehicle for selected doses of orexin-A (20 pmol), MK-4305 (200 pmol), SB 334867 (2 pmol) and EMPA (200 pmol) in the present study did not alter baseline fluorescence intensity. Doses of orexin-A higher than 20 pmol, MK-4305 higher than 200 pmol, SB 334867 higher than 2 pmol and EMPA higher than 200 pmol were not used in the present study because these doses might enhance neuronal fluorescence intensity through non-specific mechanisms that include influences on osmotic pressure.

The concentration of MK-4305 (200 pmol: 100 nM in the analytical chamber) was based on determination of the IC_{50} (8.8-12.0 nM) of this ligand to show sufficient affinity for both OX_1 and OX_2 receptors.[20] Determination of the IC_{50} (approximately 63 nM) of SB 334867 using recombinant OX_1 receptors indicated that this compound binds OX_1 receptors with nM affinity, but could bind to OX_2 receptors if high concentrations were used;[21] therefore, in order to minimize possible binding to OX_2 receptors, 2 pmol of SB 334867 (1 nM in the analytical chamber) was used to examine the roles of OX_1 receptors. Determination of the IC_{50} (7.9-8.8 nM) of EMPA using recombinant OX receptors indicated that this antagonist shows high selectivity for OX_2 over OX_1 receptors (>900-fold) and that tritium-labelled EMPA was displaced from OX_2 receptors only when extremely high concentrations of SB 334867 were used;[22] therefore, we used 200 pmol EMPA (100 nM in the

analytical chamber) to examine the roles of OX_2 receptors.

Statistical analysis

Data were expressed as means ± S.E.M. Comparisons of paw withdrawal response and relative fluorescence values were carried out using Kruskal-Wallis non-parametric analysis of variance (ANOVA) followed by post hoc Steel-Dwass test where appropriate. Comparisons of paw withdrawal threshold were carried out using one-way ANOVA followed by post hoc Scheffé's test where appropriate. A probability of $P < 0.05$ was taken to be statistically significant.

Results

Sciatic nerve ligation induces an increase in paw withdrawal response and a decrease in paw withdrawal threshold to tactile stimulation

Sciatic nerve ligation increased the mean paw withdrawal response to tactile stimulation applied by a filament with 10 g bending force and this effect lasted over an observation period of seven days (Kruskal-Wallis test, $P < 0.01$; Fig. 1A). Post hoc Steel-Dwass test revealed that responses obtained on day 1 to day 7 each differed from baseline (day 0) paw withdrawal responses ($P < 0.01$). Post hoc Steel-Dwass test also showed that paw withdrawal responses obtained across days 4 to 7 did not differ from each other. Baseline paw withdrawal responses of control rats to the tactile stimulus applied by the filament with 10 g bending force remained unchanged throughout the observation period (from day 0 to day 7; Fig. 1A).

Sciatic nerve ligation increased the mean paw withdrawal response to tactile stimulation applied by a filament with 26 g bending force and this effect lasted over an observation period of seven days (Kruskal-Wallis test, $P < 0.01$; Fig. 1B). Post hoc Steel-Dwass test revealed that responses obtained on day 1 to day 7 each differed from baseline (day 0) paw withdrawal responses ($P < 0.01$). Post hoc Steel-Dwass test also showed that paw withdrawal responses obtained across days 2 to 7 did not differ from each other. Baseline paw withdrawal responses of control rats to the tactile stimulus applied by the filament with 26 g bending force remained unchanged throughout the observation period (from day 0 to day 7; Fig. 1B).

Sciatic nerve ligation decreased the mean paw withdrawal threshold and this effect lasted over an observation period of 14 days (one-way ANOVA, $P < 0.01$; Fig. 1C). Post hoc Scheffé's test revealed that thresholds obtained on day 1 to day 7 and day 14 each differed from baseline (day 0) paw withdrawal threshold ($P < 0.01$).

Post hoc Scheffé's test also showed that paw withdrawal thresholds obtained across days 3 to 7 and day 14 did not differ from each other. Paw withdrawal thresholds of control rats remained unchanged throughout the observation period (from day 0 to day 14; Fig. 1C).

MK-4305 or EMPA, but not SB 334867, counteracts orexin-A-induced inhibition of KCl-provoked increases in relative fluorescence intensity in isolated neurons from sciatic nerve ligated rats

The approximate diameters of analysed neurons derived from control and sciatic nerve ligated rats were 23.1 ± 1.0 μm (n = 12) and 23.5 ± 0.6 μm (n = 31), respectively.

KCl (25 mM) increased relative fluorescence intensity in isolated neurons derived from DRG of control rats to 2.3 ± 0.1 (n = 5) and this effect was not influenced by orexin-A (20 pmol, n = 5; Figs. 2A and B). KCl (25 mM) similarly increased relative fluorescence intensity in isolated neurons derived from DRG of sciatic nerve ligation rats to 2.4 ± 0.2 (n = 6; Fig. 3A) but this effect was now inhibited by orexin-A (20 pmol) to 1.5 ± 0.04 (n = 6, Kruskal-Wallis test, $P < 0.01$; Figs. 3A and B). The inhibitory effect of orexin-A on KCl (25 mM)-provoked increase in relative fluorescence intensity in neurons from sciatic nerve-ligated rats (n = 6) was antagonised by co-administration of MK-4305 (200 pmol, n = 6, Kruskal-Wallis test, $P < 0.01$; Figs. 3A and B) or EMPA (200 pmol, n = 6, Kruskal-Wallis test, $P < 0.01$; Figs. 3A and B), but not by SB 334867 (2 pmol, n = 6; Figs. 3A and B). Post hoc Steel-Dwass tests revealed that the effect of orexin-A differed from vehicle ($P < 0.05$) and that the effects of co-administration of orexin-A and MK-4305 or orexin-A and EMPA differed from the effect of orexin-A alone ($P < 0.05$).

EMPA counteracts orexin-A-induced inhibition of KCl-provoked increases in relative fluorescence intensity in isolated neurons from carrageenan-treated rats

The approximate diameters of analysed neurons derived from control and carrageenan-treated rats were 23.3 ± 0.7 μm (n = 20) and 23.1 ± 0.9 μm (n = 16), respectively.

KCl (25 mM) increased relative fluorescence intensity in isolated neurons derived from DRG of control rats to 2.1 ± 0.1 and this effect was not influenced by orexin-A (20 pmol, n = 5) or EMPA (200 pmol, n = 5; data not shown).

KCl (25 mM) similarly increased relative fluorescence intensity in isolated neurons derived from DRG of carrageenan-treated rats to 2.1 ± 0.1 (Fig. 4A) but this effect was now inhibited by orexin-A (20 pmol) to 1.3 ± 0.02 (n = 6, Kruskal-Wallis test, $P < 0.01$; Fig. 4B). The inhibitory effect of orexin-A on KCl (25 mM)-provoked increase in relative fluorescence intensity in neurons from carrageenan-treated rats (n = 5) was inhibited by co-administration of EMPA (200 pmol, n = 6, Kruskal-Wallis test, $P < 0.01$; Figs. 4A and B). Post hoc Steel-Dwass tests revealed that the effect of orexin-A differed from vehicle ($P < 0.05$) and that the effect of co-administration of orexin-A and EMPA differed from the effect of orexin-A alone ($P < 0.05$).

Effects of ionomycin

Ionomycin (1 μM) strongly enhanced relative fluorescence intensity in all neuronal cells tested, to 3.4-5.0 (data not shown).

Discussion

Sciatic nerve ligation induced nociceptive behaviours in rats characterised by a stable reduction in paw withdrawal threshold and an increase in paw withdrawal response to tactile stimulation of the hind paws. Thus, behavioural analyses using filaments with 10 and 26 g bending force revealed that the paw withdrawal response to tactile stimulation was increased one day after sciatic nerve ligation (day 1). Responses to 10 and 26 g bending force remained stable from day 4 to day 7 and from day 2 to day 7, respectively. The paw withdrawal threshold decreased in response to a tactile stimulus delivered to the hind paws. The present study focused on small neuronal cells (approximately 20 μm) derived from DRG of rats on day 7 because these cells are thought to be C-fibers.[12] Ionomycin, a selective calcium ionophore that mobilizes intracellular calcium ions, strongly enhanced relative fluorescence intensity in all the cells that were examined, showing that they were viable throughout the duration of the analysis.

In elaboration of our previous report[10], which was carried out using experimental conditions similar to those of the present study, (1) infusion of a medium containing a high dose of KCl (25 mM) increased basal $[Ca^{2+}]_i$ as determined by fluorescence intensity in isolated neural cells derived from DRG of control rats, and (2) KCl-provoked increases in $[Ca^{2+}]_i$ were not affected by orexin-A infusion. Infusion of KCl also increased $[Ca^{2+}]_i$ in isolated neural cells derived from DRG of sciatic nerve ligation rats to an extent similar to that detected in nerve cells from control animals, but KCl-provoked increases in $[Ca^{2+}]_i$ were inhibited by orexin-A infusion. Thus, orexin-A was able to inhibit increases in $[Ca^{2+}]_i$ induced by KCl loading in C-fibre-like neurons derived from rats with sciatic nerve ligation-induced nociceptive behaviour, but not in such neurons from control rats with intact sciatic nerves. These results are in agreement with our previous

observations in C-fiber-like neurons derived from control rats following intra-planter administration of carrageenan to induce inflammatory stimulation.[10]

The present results indicate that orexin-A stimulates OX_2 but not OX_1 receptors on the surface of neural cells and inhibits KCl loading-induced increases in $[Ca^{2+}]_i$ in neural cells derived from rats with sciatic nerve ligation. This is because in neural cells derived from rats with sciatic nerve ligation (a) MK-4305, which blocks both OX_1 and OX_2 receptors, supressed the inhibitory effect of orexin-A on KCl loading-induced increases in $[Ca^{2+}]_i$, and (b) EMPA, which selectively blocks OX_2 receptors, but not SB 334867, which selectively blocks OX_1 receptors, counteracted the inhibitory effects of orexin-A on KCl loading-induced increases in $[Ca^{2+}]_i$. We have already reported that MK-4305 and SB 334867 show similar influences on the inhibitory effects of orexin-A on KCl-provoked increases in $[Ca^{2+}]_i$ in neurons derived from rats with intra-planter injection of carrageenan to induce inflammatory nociceptive behaviour.[10] Based on these observations, we proposed that OX_2 receptors mediate the inhibitory effects of orexin-A on increases in $[Ca^{2+}]_i$ in neural cells.

In the present study, by using a selective OX_2 receptor antagonist, we have provided neuropharmacological evidence indicating that orexin-A interacts with OX_2 receptors on C-fibre-like neuronal cells and subsequently inhibits depolarization-induced increases in $[Ca^{2+}]_i$ in such neurons derived from carrageenan treated rats. Furthermore, the present study also indicates that depolarization-induced excitation of C-fibre-like small size neurons derived from rats with either sciatic nerve ligation or intra-planter injection of carrageenan is supressed by stimulation of OX_2 but not OX_1 receptors on the surface of these neurons.

As already mentioned, orexin-A did not affect KCl-induced increases in

$[Ca^{2+}]_i$ in cells isolated from control rats, but strongly reduced such increases in neurons derived from rats with sciatic nerve ligation or treatment with carrageenan. Thus, building on our previous report,[10] the present findings further indicate that the sensitivity of C-fiber-like small neurons to orexin-A was enhanced by either sciatic nerve ligation or intra-planter administration of carrageenan. These effects of orexin-A appear to be mediated by OX_2 but not OX_1 receptors because an OX_2 but not an OX_1 receptor antagonist suppressed the inhibitory effect of orexin-A on KCl-provoked increases in $[Ca^{2+}]_i$ of isolated neurons derived from animal models of chronic pain (see above).

As mentioned in the Introduction, spinal OX_1 receptors have been shown to mediate the inhibitory effects of intra-thecally administered orexin-A on experimentally-induced mechanical allodynia and diabetic neuropathic pain of rats.[7] In neonatal rats, OX_2 receptors have been reported to reduce spinal nociceptive transmission.[23] Therefore, in order to investigate characteristics of spinal OX_2 receptor function in animals with experimentally-induced neuropathic pain, further studies should analyse the effects of intra-thecal administration of orexin-A and/or the OX_2 receptor antagonist EMPA on nociceptive behaviour in rats with sciatic nerve ligation.

On this basis we propose that decreased stimulation of OX_2 receptors by endogenous orexin-A and/or orexin-B induces supersensitivity in OX_2 receptors on neurons derived from rats with sciatic nerve ligation and treatment with carrageenan. The spinal cord is known to receive a prominent neural projection containing orexin that originates from the hypothalamus.[24] Also, central terminals of primary afferents in the spinal cord are also known to contain orexin receptors that could be activated by orexin-A and/or orexin-B released from such hypothalamo-spinal fibres.[6] Furthermore, these orexin-containing projections from the hypothalamus are

suggested to work as a descending inhibitory system for spinal nociceptive neural transmission.[6] Taken together, our previous report[10] and the present findings extend an earlier suggestion by Bingham et al.[6] that sciatic nerve ligation and intra-planter injections of carrageenan may inhibit a descending orexinergic inhibitory system that involves OX_2 receptors on neural cells derived from dorsal root ganglia. It has been suggested that mechanisms provoking inflammatory nociceptive pain and neuropathic pain are not identical, since opioids could alleviate inflammatory nociceptive pain, but often failed to alter neuropathic pain. In contrast to these assumptions, our present and previous findings imply that both inflammatory and neuropathic pain may induce an identical increase in sensitivity of orexin-A to C-fibers. Given that OX_2 receptors are likely to mediate these effects of orexin-A (see above), overview leads us to suggest that stimulation of OX_2 receptors on C-fibers may reduce not only inflammatory nociceptive pain but also neuropathic pain.

Interestingly, pharmaco-behavioural experiments using carrageenan-treated rats and diabetic rats that are known as animal models of either inflammatory pain[7] or neropathic pain,[8] respectively, have shown that spinally applied orexin-A induced anti-nociceptive effects through spinal OX_1 but not OX_2 receptors. The detailed synaptic location of these OX_1 receptors in spinal nociceptive and/or anti-nociceptive neural transmission is still unknown. However, the present findings, that orexin-A inhibited excitation of C-fiber-like neural cells via OX_2 but not OX_1 receptors, indicate that OX_1 receptors putatively localised on central terminals of primary sensory neurons are not involved in the production of spinally applied orexin-A-induced anti-nociceptive effects. In agreement with such a mechanism, OX_2 but not OX_1 receptors have been shown to play an inhibitory role in the spinal nociceptive transmission of neonatal rats.[21]

It has been suggested that orexin-A stimulates feeding.[4] Orexin-A originating from lateral hypothalamic neurones projecting to spinal areas could depolarise sympathetic preganglionic neurones in spinal cord slices through OX_1 receptor-mediated mechanisms.[5] Additionally, orexin-A is known to reduce growth hormone secretion in rats[25] and may also inhibit nociceptive neural transmission in the spinal cord from neonatal rats through OX_2 receptor-mediated mechanisms.[23] Our studies show that orexin-A may inhibit neural excitability in C-fibre-like neurons derived from experimental animals in chronic pain models through OX_2 receptor-mediated mechanisms.

In summary, the present results show that orexin-A inhibits KCl loading-induced increases in $[Ca^{2+}]_i$ in C-fiber-like small neurons derived from DRG of rats with seven-day sciatic nerve ligation-induced nociceptive behaviour. These results constitute neuropharmacological evidence that OX_2 but not OX_1 receptors mediate the inhibitory effects of orexin-A on KCl-induced increases in $[Ca^{2+}]_i$ in neurons derived from DRG of rats showing nociceptive behaviour induced by sciatic nerve ligation or intra-planter carrageenan administration. The present study suggests that neuropathic and inflammatory pain may (a) decrease OX_2 receptor-mediated input on C-fiber-like small neurons by endogenous orexin-A and/or orexin-B and (b) subsequently induce supersensitivity in these OX_2 receptors.

Acknowledgements

The authors thank Prof Drs Tsutomu Suzuki and Minoru Narita and Dr Yusuke Hamada, who enabled us to carry out sciatic nerve ligation and assess nociceptive behaviours of rodents.

The authors also appreciate critical reading of the manuscript by Prof Dr John L. Waddington.

The authors would like to thank Dr. Masataka Kimura for his excellent technical assistance and Prof Drs. Noboru Kuboyama and Koh Shibutani for their valuable advice and comments on this research.

Funding

Grant-in-Aid for Scientific Research (C) (#17K11858) from the Ministry of Education, Culture, Sports, Science and Technology, Japan
The Private University Research Branding Project from MEXT, Japan
Grants from Suzuki Fund, Nihon University School of Dentistry at Matsudo
Grants from Research Institute of Oral Science, Nihon University School of Dentistry at Matsudo

Disclosure

Conflict of interest

The authors report no conflict of interest.

Approval of the research protocol by an institutional review board.

n/a

Informed consent

n/a

Registry and the Registration No. of the study/trial

n/a

Animal Studies

All experiments were approved by the Animal Experimentation Committee of Nihon University School of Dentistry at Matsudo.

Author contributions

Y.M., M.I. and Y.A. were encouraged to analyse mechanisms of orexin-A's effects on the potassium chloride-induced increases in intraneuronal calcium ion levels by T.S. M.I. carried out behavioural and neuropharmacological experiments. Y.M., M.I. Y.A. and T.S. evaluated the analytical methods and findings. All authors discussed the results and contributed to the final manuscript.

References

1. de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, et al. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc Natl Acad Sci USA. 1998 Jan 6; 95(1): 322-7.

2. Cluderay JE, Harrison DC, Hervieu GJ. Protein distribution of the orexin-2 receptor in the rat central nervous system. Regul Pept. 2002 Mar 15; 104(1-3): 131-44.

3. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, et al. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell. 1998 Feb 20; 92(4): 573-85.

4. Baranowska B, Baranowska-Bik A, Bik W, Martynska L. The role of leptin and orexins in the dysfunction of hypothalamo-pituitary-gonadal regulation and in the mechanism of hyperactivity in patients with anorexia nervosa. Neuro Endocrinol Lett. 2008 Feb; 29(1): 37-40.

5. van den Top M, Nolan MF, Lee K, Richardson PJ, Buijs RM, Davies CH, et al. Orexins induce increased excitability and synchronisation of rat sympathetic preganglionic neurones. J Physiol. 2003 Jun 15; 549(Pt 3):809-21.

6. Bingham S, Davey PT, Babbs AJ, Irving EA, Sammons MJ, Wyles M, et al. Orexin-A, an hypothalamic peptide with analgesic properties. Pain. 2001 May;

92(1-2): 81-90.

7. Yamamoto T, Saito O, Shono K, Hirasawa S. Activation of spinal orexin-1 receptor produces anti-allodynic effect in the rat carrageenan test. Eur J Pharmacol. 2003 Nov 28; 481(2-3):175-80.

8. Kajiyama S, Kawamoto M, Shiraishi S, Gaus S, Matsunaga A, Suyama H, et al. Spinal orexin-1 receptors mediate anti-hyperalgesic effects of intrathecally-administered orexins in diabetic neuropathic pain model rats. Brain Res. 2005 May 17; 1044(1): 76-86.

9. Hunt SP, Mantyh PW. The molecular dynamics of pain control. Nat Rev Neurosci. 2001 Feb; 2(2): 83-91.

10. Ishikawa M, Aono Y, Saigusa T. Role of orexin receptor subtypes in the inhibitory effects of orexin-A on potassium chloride-induced increases in intracellular calcium ion levels in neurons derived from dorsal root ganglion of carrageenan-treated rats. J Oral Sci. 2017 Dec 27; 59(4): 557-564.

11. Martínez-Navarro M, Maldonado R, Baños J. Why mu-opioid agonists have less analgesic efficacy in neuropathic pain? Eur J Pain. 2019 Mar; 23(3):435-454.

12. Everill B, Cummins TR, Waxman SG, Kocsis JD. Sodium currents of large (Abeta-type) adult cutaneous afferent dorsal root ganglion neurons display rapid recovery from inactivation before and after axotomy. Neuroscience. 2001; 106(1):161-9. 11.

13. Kim SH, Chung JM. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain. 1992 Sep; 50(3): 355-63.

14. Kayser V, Guilbaud G. Local and remote modifications of nociceptive sensitivity during carrageenin-induced inflammation in the rat. Pain. 1987 Jan; 28(1): 99-107.

15. Callander GE, Olorunda M, Monna D, Schuepbach E, Langenegger D, Betschart C, et al. Kinetic properties of "dual" orexin receptor antagonists at OX1R and OX2R orexin receptors. Front Neurosci. 2013 Dec 3; 7: 230.

16. Kotani A, Ikeda H, Koshikawa N, Cools AR. Role of orexin receptors in the nucleus accumbens in dopamine-dependent turning behaviour of rats. Neuropharmacology. 2008 Mar; 54(3): 613-9.

17. Sadeghi S, Reisi Z, Azhdari-Zarmehri H, Haghparast A. Involvement of orexin-1 receptors in the ventral tegmental area and the nucleus accumbens in antinociception induced by lateral hypothalamus stimulation in rats. Pharmacol Biochem Behav. 2013 Apr; 105: 193-8.

18. Yan JA, Ge L, Huang W, Song B, Chen XW, Yu ZP. Orexin affects dorsal root ganglion neurons: a mechanism for regulating the spinal nociceptive processing. Physiol Res. 2008; 57(5): 797-800.

19. Winrow CJ, Gotter AL, Cox CD, Doran SM, Tannenbaum PL, Breslin MJ, et al. Promotion of sleep by suvorexant-a novel dual orexin receptor antagonist. J Neurogenet. 2011 Mar; 25(1-2): 52-61.

20. Beuckmann CT, Suzuki M, Ueno T, Nagaoka K, Arai T, Higashiyama H. In vitro and in silico characterization of lemborexant (E2006), a novel dual orexin receptor antagonist. J Pharmacol Exp Ther. 2017 Aug; 362(2): 287-295.

21. Smart D, Sabido-David C, Brough SJ, Jewitt F, Johns A, Porter RA, et al. SB-334867-A: the first selective orexin-1 receptor antagonist. Br J Pharmacol. 2001 Mar; 132(6): 1179-1182.

22. Malherbe P, Borroni E, Gobbi L, Knust H, Nettekoven M, Pinard E, et al. Biochemical and behavioural characterization of EMPA, a novel high-affinity, selective anta gonist for the OX(2) receptor. Br J Pharmacol. 2009 Apr; 156(8): 1326-1341.

23. Shono K, Yamamoto T. Orexin-2 receptors inhibit primary afferent fiber-evoked responses of ventral roots in the neonatal rat isolated spinal cord. Brain Res. 2008 Jul 7; 1218: 97-102.

24. van den Pol AN. Hypothalamic hypocretin (orexin): robust innervation of the spinal cord. J Neurosci. 1999 Apr 15; 19(8): 3171-82.

25. Seoane LM, Tovar SA, Perez D, Mallo F, Lopez M, Señaris R, et al. Orexin A suppresses in vivo GH secretion. Eur J Endocrinol. 2004 May; 150(5): 731-6.

Figure legends

Fig. 1

(A and B) Changes in paw withdrawal in response to a tactile stimulus in rats with sciatic nerve ligation. Data are expressed as means of the following scores to evaluate paw withdrawal response: 0, no response; 1, a slow and/or slight response to the stimulus; 2, a quick withdrawal response away from the stimulus without flinching or licking; 3, an intense withdrawal response away from the stimulus with brisk flinching and/or licking. Vertical bars indicate S.E.M. The tactile stimulus was applied by using filaments with two different bending forces, namely 10 g (A) and 26 g (B). In rats with sciatic nerve ligation, the L5 and L6 spinal nerves were isolated and tightly ligated with 3-0 silk thread. In control rats, the nerve was exposed without ligation. (C) Changes in paw withdrawal threshold of rats with sciatic nerve ligation. Data are expressed as mean paw withdrawal threshold (g) of hind paws after the sciatic nerve ligation. In control rats, the nerve was exposed without ligation. Vertical bars indicate S.E.M. Asterisks in A and B indicate statistical comparisons per time point between baseline (day 0) and day 1 to day 7 that are statistically significant (post hoc Steel-Dwass test, $P < 0.05$). Asterisks in C indicate statistical comparisons per time point between baseline (day 0) and day 1 to day 14 that are statistically significant (post hoc Scheffé's test, $P < 0.05$).

Fig. 2

(A) Effects of vehicle ($n = 5$) and orexin-A (20 pmol, $n = 5$) on KCl (25 mM)-induced increases in relative fluorescence values F/F_0 measured in DRG neurons from control rats. F denotes neuronal fluorescence intensity at each time point; F_0 denotes baseline neuronal fluorescence intensity. Data are expressed as

mean changes in 1 s lapse time after addition of KCl (25 mM) to the bath solution in the analytical chamber. Vertical bars indicate S.E.M. KCl was applied to the bath solution in the analytical chamber at 0 s. Vehicle and orexin-A were applied to the bath solution in the analytical chamber 60 s before the KCl treatment. (B) Mean values of maximum F/F_0 shown in Fig. 2A. Vertical bars indicate S.E.M.

Fig. 3

(A) Effects of vehicle (n = 5) and orexin-A (20 pmol, n = 6) with or without MK-4305 (200 pmol, n = 5), SB 334867 (2 pmol, n = 5) or EMPA (200 pmol, n = 5) on KCl (25 mM)-induced increases in relative fluorescence values F/F_0 measured in DRG neurons from rats with sciatic nerve ligation. F denotes neuronal fluorescence intensity at each time point; F_0 denotes baseline neuronal fluorescence intensity. Data are expressed as mean changes in 1 s lapse time after addition of KCl (25 mM) to the bath solution in the analytical chamber. Vertical bars indicate S.E.M. KCl was applied to the bath solution in the analytical chamber at 0 s. Vehicle, orexin-A, orexin-A + MK-4305, orexin-A + SB 334867 or orexin-A + EMPA were applied to the bath solution in the analytical chamber 60 s before the KCl treatment. (B) Mean values of maximum F/F_0 shown in Fig. 3A. Vertical bars indicate S.E.M. Asterisks indicate statistical comparisons between vehicle and drug treatments that are statistically significant (post hoc Steel-Dwass test, $P < 0.05$).

Fig. 4

(A) Effects of vehicle (n = 6) and orexin-A (20 pmol, n = 5) with or without EMPA (200 pmol, n = 6) on KCl (25 mM)-induced increase in relative fluorescence values F/F_0 measured in DRG neurons from carrageenan-treated rats. F denotes neuronal fluorescence intensity at each time point; F_0 denotes baseline neuronal fluorescence

intensity. Data are expressed as mean changes in 1 s lapse time after addition of KCl (25 mM) to the bath solution in the analytical chamber. Vertical bars indicate S.E.M. KCl was applied to the bath solution in the analytical chamber at 0 s. Vehicle, orexin-A or orexin-A + EMPA were applied to the bath solution in the analytical chamber 60 s before the KCl treatment. (B) Mean values of maximum F/F_0 shown in Fig. 4A. Vertical bars indicate S.E.M. Asterisks indicate statistical comparisons between vehicle and drug treatments that are statistically significant (post hoc Steel-Dwass test, $P < 0.05$).

Supporting information list

NPPR Yamaguchi et al.xlsx