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Original Article

Yokukansan (Kampo medicinal formula) prevents the development of morphine tolerance by inhibiting the secretion of orexin A



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ABSTRACT

Background: Yokukansan (YKS), a traditional herbal (Kampo) medicine consisting of seven herbs, is effective in the treatment of pain disorders, such as headache, postherpetic neuralgia, fibromyalgia, and trigeminal neuralgia, and we have previously shown it to be effective against morphine analgesic tolerance in rats. It has been reported that orexin receptor antagonists prevent the development of morphine tolerance and that YKS inhibits the secretion of orexin A in the hypothalamus. This study examined whether the inhibition of the secretion of orexin A by YKS is one mechanism underlying its effect against morphine analgesic tolerance.

Methods: Male Wistar rats were administered a subcutaneous injection of morphine hydrochloride (10 mg/kg/day) for 5 days. One group was preadministered YKS, starting 3 days before the morphine. The withdrawal latency following thermal stimulation was measured daily using a hot plate test. On day 5, the levels of orexin A in the plasma and the midbrain were measured, and the appearance of activated astrocytes in the midbrain was examined by immunofluorescence staining.

Results: The preadministration of YKS prevented the development of morphine tolerance. The repeated administration of morphine significantly increased the plasma and midbrain levels of orexin A and the activation of astrocytes. These increases were significantly inhibited by the preadministration of YKS.

Conclusion: These results suggest that the preadministration of YKS attenuated the development of antinociceptive morphine tolerance and that the inhibition of orexin A secretion may be one mechanism underlying this phenomenon.

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1. Introduction

Morphine is a powerful opioid analgesic widely used for relieving severe pain, such as the pain associated with cancer and surgery. However, its repeated administration may lead to dependence and the development of antinociceptive tolerance. Morphine tolerance is a complex physiological response involving glial cell activity,^{1–3} neuroinflammation,^{4–7} opioid receptor desensitization,⁸ and the activation of spinal N-methyl-D-aspartate receptors.⁹ In recent years, it has been reported that orexins are also involved in the development of antinociceptive tolerance.^{10–12}

Orexins (also called hypocretins) are neuropeptides. Orexinergic neurons are mainly expressed in the lateral and perifornical areas of the posterior hypothalamus, with their axons widely distributed throughout the central nervous system, apart from in the cerebellum.¹³ Orexins A and B are derived from the same precursor peptide, prepro-orexin,¹⁴ and bind to the orphan G-protein coupled receptor orexin-1 receptor (OX1R), which is highly selective for orexin A, and to the orexin-2 receptor (OX2R), which is a nonselective receptor for both orexins A and B.¹³ Orexins are involved in the modulation of many physiological processes, including sleep, arousal, feeding, and metabolism,¹⁵ as well as in the autonomic regulation of the cardiovascular,¹⁶ respiratory,¹⁷ and neuroendocrine systems.¹⁸ Many studies have indicated that orexin A also exhibits antinociceptive effects in the brain and spinal cord, based on different types of pain models with thermal, chemical,^{19,20} and neuropathic nociception.^{21,22} Conversely, orexin and OX1R may be involved in the development of tolerance to the analgesic effects of morphine. Studies have shown that pretreatment with an OX1R antagonist inhibited the development of morphine tolerance.^{10–12}

Yokukansan (YKS) is a traditional herbal (Kampo) medicine that comprises seven herbs (Table 1). It is administered to patients who show symptoms such as emotional irritability, neurosis, and insomnia and to infants who suffer from night crying and convulsions.²³ Recently, it has been reported that YKS is also effective against pain disorders, including headache, postherpetic neuralgia, fibromyalgia, and trigeminal neuralgia.^{24,25} Studies have demonstrated the antinociceptive effects of YKS in mice models with visceral pain¹¹ and rat models with chronic constriction injury.^{26–28} We have previously reported that preadministration of YKS attenuated the development of antinociceptive morphine tolerance, and that the suppression of spinal glial cell activation

may be one mechanism underlying this phenomenon.²⁹ We also showed that the administration of YKS reduced the secretion of orexin A in a rat stress model.³⁰

In the present study, therefore, we investigated whether inhibition of the secretion of orexin A is involved in the effect of YKS against morphine analgesic tolerance.

2. Methods

2.1. Animals

Male Wistar rats (7–8 weeks old) were purchased from Nippon Bio-Supp. Center (Tokyo, Japan). During the experimental period, the animals were housed in standard plastic cages (W 24 × L 40 × H 20 cm) and were kept in our animal facility at 25 ± 2°C and 55% ± 5% humidity, with a light/dark cycle of 12 hours/12 hours. Food (CLEA Japan, CE-2, Tokyo, Japan), and water was provided *ad libitum*. The experiments were performed in accordance with the guidelines of the Committee of Animal Care and Welfare of Showa University. All experimental procedures were approved by the Committee of Animal Care and Welfare of Showa University (certificate number: 07062).

2.2. Administration of drugs

The dry powdered extracts of YKS (Lot No. 2110054010) used in the present study were supplied by Tsumura & Co. (Tokyo, Japan). The seven herbs comprising YKS (Table 1) were mixed and extracted with purified water at 95.1°C for 1 hour; the soluble extract was separated from the insoluble waste and concentrated by removing water under reduced pressure. The YKS was dissolved in distilled water and orally administered (1.0 g/kg/day). This dose was chosen on the basis of effective doses of YKS recommended in previous reports.³¹ The rats which were not treated with YKS received water instead.

Morphine hydrochloride (T1-02591; Takeda Chemical Industries, Osaka, Japan) was dissolved in physiological saline and injected subcutaneously. The rats not treated with morphine were administered only saline.

2.3. Influence of morphine administration on orexin A secretion

The influence of a single administration of morphine on orexin A secretion was examined. Eighteen rats were randomly divided into three groups (each *n* = 6): Control, which were administered physiological saline; Mor (2 mg), subcutaneously administered with morphine at 2 mg/kg; and Mor (10 mg), subcutaneously administered with morphine at 10 mg/kg. At 30 minutes after dosing, the rats were anesthetized with intraperitoneally administered pentobarbital sodium (50 mg/kg) (Somnopentyl; Kyoritsu Seiyaku, Tokyo, Japan). Blood samples were taken from the inferior vena cava, and midbrain samples were then removed. To avoid the influence of daily fluctuations, all samples were obtained between 1:00 and 3:00 PM. The blood samples were centrifuged at 4°C and 3000 rpm for 10 minutes and the supernatants collected. The midbrain samples were mashed homogeneously with a

Table 1 – The Component Galenicals of Yokukansan

Component galenicals of Yokukansan (YKS)

<i>Uncariae cum Uncis ramulus</i>	3.0 g
<i>Cnidii rhizoma</i>	3.0 g
<i>Bupleuri radix</i>	2.0 g
<i>Atratyloidis Lanceae rhizoma</i>	4.0 g
<i>Poria</i>	4.0 g
<i>Angelicae radix</i>	3.0 g
<i>Glycyrrhizae radix</i>	1.5 g

The weights indicate the relative amounts mixed.

pestle in 10 volumes (v/w) of a neuronal protein extraction reagent (N-PER, Thermo Scientific, Rockford, IL, USA) and incubated for 10 minutes on ice. The homogenate was centrifuged at $10,000 \times g$ for 10 minutes and the supernatant collected. All samples were subsequently stored at -80°C until the analysis. Orexin A levels were measured using an enzyme-linked immunosorbent assay kit (EKE-003-30; Phoenix Pharmaceuticals, Burlingame, CA, USA).

2.4. Inhibitory effect of YKS on morphine tolerance

The withdrawal latency was assessed using a hot plate test to investigate YKS's inhibitory effect on the development of antinociceptive tolerance to morphine.²⁹ Twenty-four rats were randomly divided into three groups (each $n=8$): Control; Mor, treated with morphine; and YKS + Mor, preadministered with YKS and then treated with morphine. The administration of YKS (between 11:30 AM and 1:30 PM) was started 3 days prior to the morphine injection in the YKS + Mor group because a single administration did not work effectively in our previous study.²⁹ Nakagawa et al³¹ also preadministered YKS to morphine tolerance model mice. Rats in the Mor and YKS + Mor groups received a subcutaneous injection of morphine hydrochloride (10 mg/kg) once daily (between 12:30 and 2:30 PM) for 5 days, whereas those in the Control group received a subcutaneous injection of physiological saline. At 30 minutes after the injections (between 1:00 and 3:00 PM), the rats were placed on a hot plate (35100 Hot Cold Plate: Ugo Basile, Varese, Italy) with the temperature set at 55°C . The latency up to the first sign of paw licking or jumping in response to the heat was measured, with 10 seconds considered the cutoff point to avoid tissue damage.

2.5. Orexin A levels in plasma and the midbrain following the repeated administration of morphine

As described in Section 2.4, twenty-four rats were randomly divided into a Control group, a Mor group, and a YKS + Mor group. On day 5, the rats were anesthetized with intraperitoneally administered pentobarbital sodium (50 mg/kg) (Somnopentyl; Kyoritsu Seiyaku), and blood samples (from 24 rats) and midbrain samples (from 15 rats) were taken 30 minutes after the injection of morphine (between 1:00 and 3:00 PM). The orexin A levels were measured using the method described in Section 2.3.

2.6. Immunofluorescence staining

Midbrain astrocytes have also been implicated in the development of morphine tolerance.³² In the present study, the appearance of activated astrocytes in the periaqueductal gray (PAG) and the presence of OX1R in the astrocytes were investigated using immunofluorescence staining, with glial fibrillary acidic protein (GFAP) staining used to label the astrocytes.¹ Fifteen rats were randomly divided into a Control group, a Mor group, and a YKS + Mor group. On day 5, 30 minutes after the morphine or saline injection (between 1:00 and 3:00 PM), the rats were intraperitoneally anesthetized with pentobarbital sodium (50 mg/kg) and were intracardially perfused with phosphate buffered saline at pH 7.4 until all

the blood had been removed from the system. After perfusion with 4% paraformaldehyde in 0.1M phosphate buffered saline, the midbrain samples were harvested. The tissue specimens were embedded in optimum cutting temperature compound (Tissue-Tek OCT, Sakura Finetek, Torrance, CA, USA), frozen and then cut into 20- μm sections using a cryostat (CM3050S; Leica Biosystems, Nussloch, Germany). The sections were incubated overnight at 4°C with mouse anti-GFAP antibody (G3893; 1:500, Sigma-Aldrich, St. Louis, MO, USA) and rabbit anti-OX1R antibody (LC-C385249; 1:400, Lifespan Biosciences, Seattle, WA, USA). These were then incubated for 2 hours with fluorophore-tagged secondary antibodies: donkey anti-mouse Alexa Fluor 555 (1:1000, Thermo Fisher Scientific, Waltham, MA, USA) for GFAP, and donkey anti-rabbit Alexa Fluor 488 (1:1000, Thermo Fisher Scientific) for OX1R. The nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole, 1:1000, Thermo Fisher Scientific). The samples were observed using a confocal laser scanning fluorescence microscope (FV1000D, Olympus, Tokyo, Japan), and the optical densities of the GFAP immunoreactive staining were measured using the FV10-AW software program (Olympus). All values were reported as the mean of five micrographs per rat.

2.7. Statistical analysis

The experimental data are presented as mean \pm standard deviation. The statistical significance of differences between groups was evaluated by one-way analysis of variance tests, with posthoc comparisons between the groups performed using Tukey's test. p -values < 0.05 were considered to be statistically significant.

3. Results

3.1. Influence of morphine on orexin A secretion

The influence of the administration of morphine on orexin A secretion was investigated. The subcutaneous administration of morphine in the Mor (2 mg) and Mor (10 mg) groups significantly increased plasma orexin A levels compared with that in the control group (Control: 113.02 ± 24.41 pg/mL; Mor (2 mg): 157.46 ± 31.02 pg/mL; Mor (10 mg): 169.92 ± 18.16 pg/mL); however, no significant difference was observed between the Mor (2 mg) and Mor (10 mg) groups (Fig. 1a). The values in the midbrain were corrected for the protein concentration, measured using Bradford protein assays, and are expressed as the ratio of orexin A to total protein.³³ As with the plasma results, the administration of morphine significantly increased the orexin A levels compared with the level in the control group (Control: 35.12 ± 32.56 pg/mL/protein; Mor (2 mg): 120.92 ± 74.80 pg/mL/protein; Mor (10 mg): 119.66 ± 47.31 pg/mL/protein) (Fig. 1b).

3.2. Inhibitory effect of YKS on morphine antinociceptive tolerance

We examined the effect of YKS on morphine antinociceptive tolerance. On day 4, the withdrawal latency started to decrease in the Mor group. On day 5, the withdrawal latency in the

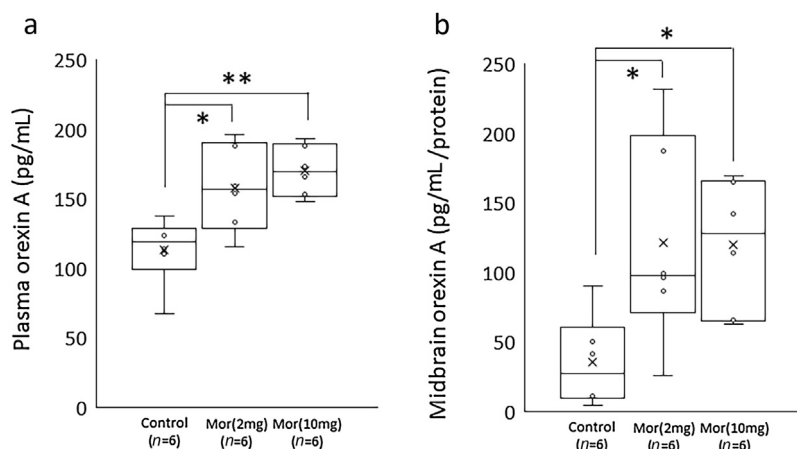


Fig. 1 – Influence of morphine administration on orexin A secretion. The levels of orexin A in plasma (a) and the midbrain (b) following a single subcutaneous injection of morphine at doses of 2 mg/kg [Mor (2 mg), $n=6$] and 10 mg/kg [Mor (10 mg), $n=6$]. Morphine significantly increased the levels in plasma and the midbrain compared with the levels in the Control group ($n=6$). Horizontal lines within boxes denote median values, and x-marks denote mean values. * $p < 0.05$ (vs. Control), ** $p < 0.01$ (vs. Control).

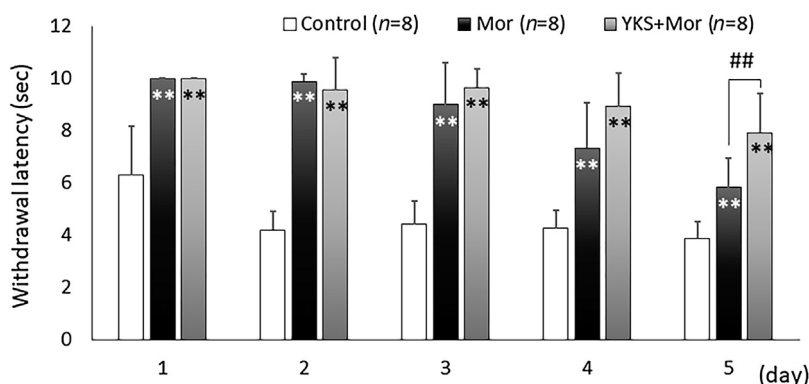


Fig. 2 – Inhibitory effect of Yokukansan (YKS) on morphine tolerance using the hot plate test. On day 4, the thermal withdrawal latency started decreasing in the group treated with morphine (Mor, $n=8$), and on day 5, the decrease was significantly inhibited in the group treated with YKS and morphine (YKS + Mor, $n=8$) in comparison with that in the Mor group. This indicated that administration of YKS could prevent the development of morphine tolerance. ** $p < 0.01$ (vs. Control), ## $p < 0.01$ (vs. Mor).

Mor group (5.83 ± 1.14 s) was significantly lower than that in the YKS + Mor group (7.93 ± 1.52 s) ($p < 0.01$) (Fig. 2). Thus, the preadministration of YKS significantly inhibited the development of morphine tolerance.

3.3. Changes in orexin A secretion following the daily administration of morphine and YKS

On day 5, changes in orexin A secretion were investigated. The daily administration of morphine had significantly increased plasma orexin A levels compared with those in the control group (Control: 93.89 ± 38.64 pg/mL; Mor: 135.93 ± 28.36 pg/mL); however, preadministration of YKS significantly inhibited this increase (YKS + Mor: 85.95 ± 24.31 pg/mL) (Fig. 3a). The levels in the midbrain also significantly increased in the group-administered morphine compared with in the control group (Control: 63.73 ± 15.05 pg/mL/protein; Mor: 181.18 ± 76.46 pg/mL/protein); however, the level of orexin A

secretion in the YKS + Mor group was significantly lower than that in the Mor group (YKS + Mor: 99.29 ± 9.14 pg/mL/protein) (Fig. 3b). Thus, preadministration of YKS inhibited the increase of orexin A secretion caused by the morphine injections.

3.4. Immunofluorescence staining in the PAG

3.4.1. Changes in astrocytes in the PAG

GFAP immunoreactivity was assessed in the PAG (Fig. 4a-i). The Mor group showed an increase in the number of GFAP (+) cells (Fig. 4b,e) compared with the control group (Fig. 4a,d); however, this increase was inhibited in the YKS + Mor group (Fig. 4c,f). The GFAP immunoreactivity (IR) values for the images (magnification, 200 \times) were then expressed as optical densities (Fig. 4j). The GFAP level was significantly higher in the Mor group than in the Control group (198.83 ± 65.24 vs. 98.13 ± 30.24 IR density, $p < 0.01$). This increase was significantly inhibited in the YKS + Mor group (112.38 ± 18.86 IR density; $p < 0.01$). Furthermore, astrogliosis was confirmed in

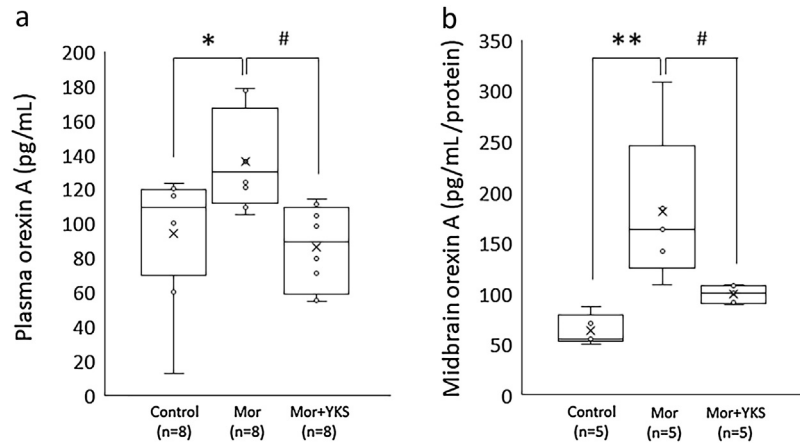


Fig. 3 – Orexin A levels in plasma and the midbrain following the repeated administration of morphine. The levels of orexin A in plasma (a) and the midbrain (b) following the repeated administrations of morphine (Mor, $n = 8$) or Yokukansan and morphine (YKS + Mor, $n = 8$) for 5 days. Morphine significantly increased the levels of orexin A in both plasma and the midbrain compared with the levels in the Control group ($n = 8$); however, the increases were significantly inhibited by the administration of Yokukansan. Horizontal lines within boxes denote median values, and x-marks denote mean values. * $p < 0.05$, ** $p < 0.01$ (Control vs. Mor). # $p < 0.05$ (Mor vs. Mor + YKS).

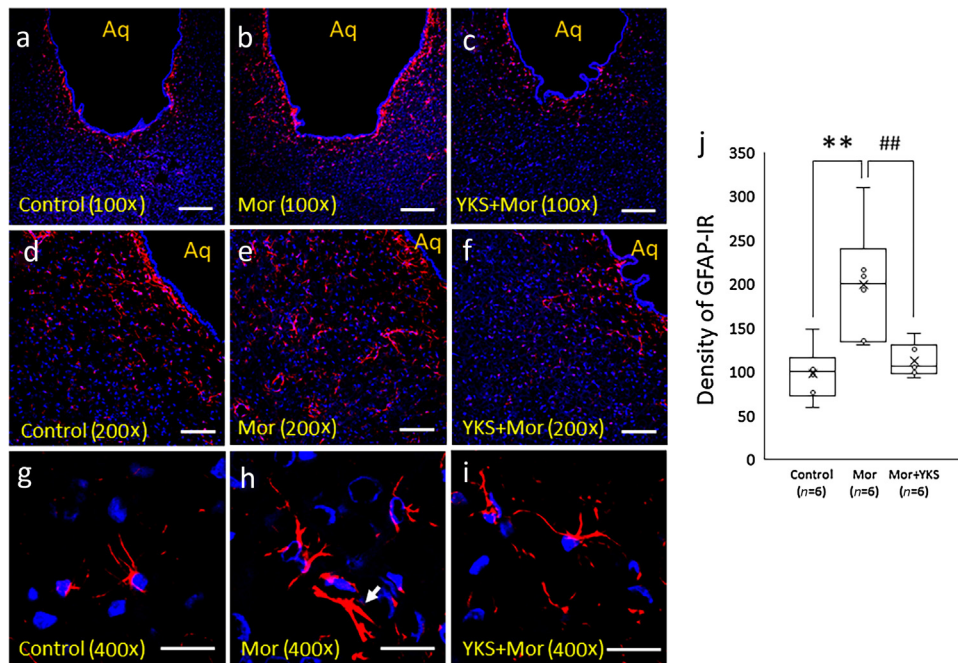


Fig. 4 – Inhibitory effect of Yokukansan on astrocyte activation induced by morphine treatment. (Magnifications: a–c: 100 \times , scale bar = 200 μm ; d–f: 200 \times , scale bar = 100 μm ; g–i: 400 \times , scale bar = 25 μm .) Glial fibrillary acidic protein (GFAP) staining in the periaqueductal gray on day 5 following morphine and Yokukansan treatment (a–i), and the fluorescence density quantification of GFAP immune reactivity (j). GFAP expression increased significantly in the group administered morphine (Mor; b,e) compared with in the Control group (a,d), but this increase was inhibited in the group treated with Yokukansan and morphine (YKS + Mor; c,f). In the Mor group, astrogliosis (white arrow) was confirmed by the intense GFAP immunoreactivity and hypertrophied astrocytes with thick processes (h); this change was suppressed in the YKS + Mor group (i). Aq: aqueduct of the midbrain, IR: immunoreactivity. Horizontal lines within boxes denote median values, and x-marks denote mean values. ** $p < 0.01$ (Control and Mor). ## $p < 0.01$ (Mor vs. Mor + YKS).

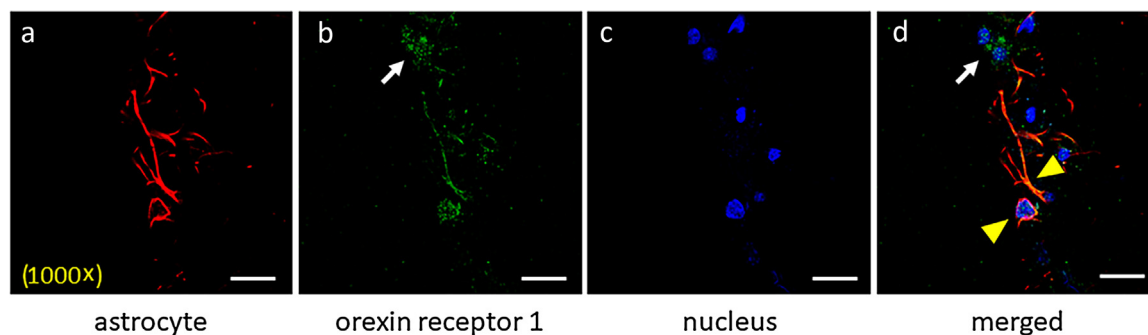


Fig. 5 – Presence of an orexin-1 receptor (OX1R) in an astrocyte. (Magnification. a–d: 1000 \times , scale bar = 20 μ m.) Red indicates the astrocyte (a), green indicates the OX1R (b), and blue indicates nuclei (c). Superimposing the images (d) confirmed the presence of OX1R in the astrocyte (yellow arrowheads in d), although parts of the OX1R were outside the astrocyte (white arrows in b and d).

images (magnitude, 400 \times) of the Mor group, identified from the intense GFAP immunoreactivity and hypertrophied astrocytes with thick processes (indicated by the white arrow in Fig. 4h). This change was suppressed in the YKS + Mor group (Fig. 4i).

3.4.2. Orexin-1 receptors in the astrocytes

We investigated whether astrocytes contained OX1Rs. Fig. 5 shows a series of images in which red indicates the astrocyte (Fig. 5a), green indicates OX1R (Fig. 5b), and blue indicates nuclei (Fig. 5c). Superimposing the three images confirmed the presence of OX1R in the astrocyte (yellow arrowheads in Fig. 5d), although parts of the OX1R were outside of the astrocyte (white arrows in Fig. 5b and d).

4. Discussion

We have previously reported that the preadministration of YKS attenuated the development of antinociceptive morphine tolerance, and that the suppression of spinal astrocyte activation may be one mechanism underlying this phenomenon.²⁹ Midbrain astrocytes have also been reported to be involved in the development of morphine tolerance.³² Furthermore, in recent years, orexin A has been implicated in the development of antinociceptive tolerance.^{10–12} In this study, therefore, we focused on astrocytes in the midbrain and investigated the involvement of orexin A in the inhibitory effect of YKS on morphine tolerance.

First, the influence of morphine administration on the levels of orexin A in plasma and the midbrain were investigated. The orexin neuron has a mu-opioid receptor and responds to morphine administration.³⁴ OX1R is involved in morphine-induced antinociceptive effects,³⁵ and the projections of orexin neuron and orexin receptors are distributed in the midbrain³⁶; we therefore hypothesized that the administration of morphine increases the levels of orexin A in plasma and the midbrain. Two doses of morphine were used: 2 mg/kg and 10 mg/kg. In animal studies, 2 mg/kg morphine is used for analgesia,³⁷ and 10 mg/kg is used to develop morphine tolerance.³² Our findings confirmed that the administration of morphine significantly increased the secretion of orexin A into the midbrain.

We then examined the effect of YKS on morphine antinociceptive tolerance. Previous studies have indicated that preadministration of YKS may inhibit the development of antinociceptive tolerance to morphine,^{29,31} so in this study the administration of YKS was started 3 days before the first injection of morphine. On day 5, the withdrawal threshold in the group preadministered YKS was significantly higher than that in the group administered morphine without the YKS. We examined pathological changes on that day and observed that orexin A secretions in plasma and the midbrain were kept at an increased level by the repeated administration of morphine, but that this increase was inhibited by the preadministration of YKS. Orexin neurons are inhibited by serotonin (5-HT), noradrenalin, and gamma-aminobutyric acid, and the action of 5-HT is transduced through the 5-HT_{1A} receptor.^{38,39} YKS comprises seven crude medicinals (Table 1) and acts as an agonist of the 5-HT_{1A} receptor; there is evidence that geissoschizine methyl ether, an alkaloid synthesized by *Uncaria cum Uncis ramulus*,^{40,41} mediates this activity. Thus, the action of *Uncaria cum Uncis ramulus* via the 5-HT_{1A} receptor may represent one of the mechanisms through which YKS exerts its inhibitory effect on orexin neurons. Future studies are required to identify crude medicinals that regulate orexin secretion as well as the associated mechanisms.

We observed that astrocytes were activated by the repeated administration of morphine, but that this activation was inhibited by the preadministration of YKS. A clinical study reported that pentoxifylline enhanced the effects of morphine after surgery, and that patients who received preoperative pentoxifylline had lower opioid requirements.⁴² Astrocyte activation in the midbrain and the development of analgesic tolerance to morphine were significantly inhibited following pretreatment with pentoxifylline in mice.³² We previously reported that YKS may inhibit the activation of astrocytes,^{28,29} although the mechanism for this is unclear. An in vitro study showed that OX1R and OX2R were expressed in cultured rat astrocytes, and orexin A stimulated the phosphorylation of extracellular signal-regulated kinase 1/2 and then induced the migration of astrocytes via its receptor, OX1R.⁴³ We confirmed that astrocytes in the midbrain also contained OX1R. These findings suggest that one mechanism for the development of analgesic tolerance may be the increased secretion of orexin

A caused by the repeated administration of morphine activating astrocytes in the midbrain, and that preadministration of YKS attenuates this development via the inhibition of orexin A secretion.

In conclusion, our findings suggest that preadministration of YKS attenuates the development of antinociceptive morphine tolerance, and that the suppression of orexin A secretion may be one mechanism underlying this phenomenon. To the best of our knowledge, this study is the first to examine the relationship between YKS and orexin A secretion in morphine tolerance. However, further studies are needed to clarify the underlying mechanisms.

Conflict of interest

The authors declare no conflict of interest.

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