Doctoral Thesis in Medicine

# Orexin receptors mediate long-term depression of excitatory synaptic transmission in the spinal cord dorsal horn

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이 논문을 의학박사학위논문으로 제출함.

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박기범의 의학박사학위논문을 인준함.

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# Abstract

**Orexin receptors mediate long-term depression of excitatory synaptic transmission in the spinal cord dorsal horn**

Neuropeptides orexin-A and -B are related to the regulation of sleep/wakefulness and feeding behaviors. Recently, the peptides have also been shown to yield antinociceptive effects in various pain models. However, it is not clear whether orexins are involved in forms of synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD), the increase and the decrease of synaptic efficacy, respectively. In the present study, we examined whether orexin receptor type 1 (OX1) and 2 (OX2) are involved in the induction or maintenance of LTD of excitatory synaptic transmission using transverse spinal cord slices of young rats. Repetitive electrical stimulation of Lissauer's tract zone at 2 Hz for 5 min (600 pulses), combined with a holding potential of -30 mV, induced LTD of the amplitude of excitatory postsynaptic currents (EPSCs) which are evoked by the activation of primary afferent fibers. The maintenance of LTD was significantly prevented by bath application of SB674042 (1 μM), an OX1 antagonist, or EMPA (1μM), an OX2 antagonist. In addition, LTD was dependent on the NMDA receptor, as the NMDA receptor antagonist D-AP5 blocked the maintenance of LTD. Our study suggests that orexins, via activation of both OX1 and OX2, play a significant role in the expression of NMDA-dependent LTD, thereby contributing to the spinal modulation of pain transmission.



Keywords : orexins; long-term synaptic depression; receptors, N-Methyl-D-Aspartate

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# Ⅰ. Introduction

Certain patterns of pre- and/or postsynaptic activities at excitatory or inhibitory synapses increase or decrease synaptic efficacy, respectively indicating long term potentiation (LTP) and long term depression (LTD), and many different patterns of activities have been studied in various regions of the brain in terms of types of synaptic plasticity induced [1]. At excitatory synapses between primary afferent fibers and spinal dorsal horn (DH) neurons, high-frequency stimulation (HFS) at 100 Hz induces LTP or LTD, both of which are dependent on the activation of the NMDA receptor [2]; while low-frequency stimulation (LFS) induces only NMDA receptor-dependent LTD in the spinal DH [3] or LTP in the lamina I spinal DH neurons projecting to periaqueductal gray [4]. In contrast, cellular models for both LTP and LTD have been developed to explain synaptic mechanisms of hyperalgesia and allodynia, the increased responses against noxious painful or non-noxious non-painful stimuli, respectively [5]. In the case of LTD, it may be related to antinociception after repetitive afferent stimulation [3]; the LFS protocol for LTD induction revealed reduced pain perception by single electrical stimulation in humans [6].

Orexin A and B act through G protein-linked receptors, the orexin receptors type 1 (OX1) and 2 (OX2). Orexins are produced in neurons in the lateral and perifornical areas of the hypothalamus and their receptors are abundant at the locus coeruleus (LC) [7]. In addition, orexin-A and orexin-B are abundant in the superficial lamina (laminae I and II) of the spinal cord [8,9]. The analgesic effect of intrathecal and intracerebroventricular injection of orexin A in the rat model of partial sciatic nerve ligation reduced

mechanical allodynia [10]. Previously, our experiment showing the effect of orexin A on excitatory synaptic transmission in the substansia gelatinosa (SG; lamina II) of the spinal dorsal horn revealed a depression of excitatory synaptic transmission activated by Aδ- and C-fibers [11]. This depression may be correlated with pain reduction by spinally-applied orexin A in inflammatory and neuropathic pain models [12,13].

In this context, it is interesting to investigate whether orexins and their receptors are involved in the induction of LTD. Therefore, we examined the association between LFS-induced LTD and OX1 or OX2 receptors in the dorsal horn of the spinal cord.

# Ⅱ. Methods

## 1. Slice preparation

Transverse spinal cord slices were obtained from Sprague Dawley rats (13–21 days old of both sexes). Slices (450 μm) were cut from lumbar 4 - 5 segments without dorsal roots. After deep anesthesia with urethane (1.5 mg/kg) administered into the intraperitoneal cavity, the lumbar spine was dissected and the spinal cord extracted and quickly placed in a petri dish filled with ice-cold Krebs' solution which was oxygenated by 95% oxygen/ 5% $CO_2$ (composition in mM: NaCl, 117; KCl, 3.6; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaH_2PO_4$, 1.2; $NaHCO_3$, 25; and glucose, 11). Transverse slices were cut by using a vibratome (Vibratome $1000^+$, Vibratome Co., St. Louis, MO) in the oxygenated cold Krebs solution. All Slices were submerged in the Krebs' solution for at least 1 hour. Further, the slice was transferred into the recording chamber, where it was perfused with Krebs' solution. The recording chamber was continuously perfused (3 ml/min) with preoxygenated Krebs' solution at room temperature (23–24 ºC).

## 2. Blind whole-cell patch clamp recordings

The SG area, which was distinguished as a translucent band in the spinal cord slice under the light microscope, was selected for recording with a 4× objective lens, as previously described [11]. Micropipettes were made by borosilicate glass (TW150F; WPI, Sarasota, FL) and used by being filled with the internal solution (composition in mM: K-gluconate, 145; NaCl, 5;

$MgCl_2$, 1; EGTA, 0.2; HEPES, 10; Tris-ATP, 2). The final tip resistance of patch pipettes was 4–6 MΩ. Recordings were made in a voltage-clamp mode using Axopatch 1D amplifier and pCLAMP 9 data acquisition software (Molecular Devices, Sunnyvale, CA). At the holding potential ($V_H$) of -30 mV or -70 mV, according to protocol, excitatory postsynaptic currents (EPSCs) were evoked by focal electrical stimulation (0.5 millisecond, 5 - 25 V; every 30 sec) to the Lissauer's tract zone with a concentric bipolar stimulating electrode (TM33CCINS; tip diameter, 3-4 μm; WPI, FL). To induce LTD, LFS protocols were applied. During LFS, recorded neurons were depolarized as indicated.

## 3. Drugs and data analysis

The drugs utilized in this study were purchased from Tocris Cookson (Ellisville, MO): [5-(2-fluorophenyl)-2-methyl-4-thiazolyl][2(S)-2-[(5-phenyl-1,3,4-oxadiazol-2-yl)methyl-1-pyrrolidinyl]methanone (SB674042), *N*-ethyl-2-[(6-methoxy-3-pyridinyl)[(2-methylphenyl)sulfonyl]amino]-*N*-(3-pyridinylmethyl)-acetamide (EMPA) and D-(-)-2-amino-5-phosphonopentanoic ac id (D-AP5). Each drug was dissolved as stock solution in either distilled water or dimethyl sulfoxide, and applied by exchanging the perfusion solution with the drug solution at a final concentration indicated. Statistics for data analysis was assessed by Student's *t*-test within the group and by ANOVA among the groups at same time points. Statistical significance was indicated as $P < 0.05$ or $P < 0.01$. Data are expressed mean ± standard error of the mean (SEM).

# Ⅲ. Results

## 1. Low-frequency stimulation-induced LTD in the spinal cord substantia gelatinosa is dependent on postsynaptic depolarization

In the spinal dorsal horn, including the SG (lamina II), LTD of excitatory synaptic transmission was induced by an LFS protocol, which consists of 900 pulses at 1 Hz in the current-clamp mode [3]. In the current clamp condition, the level of postsynaptic depolarization occurred naturally during conditioned low-frequency repetitive stimulation. In this study, which recorded synaptic responses in the voltage-clamp mode, we examined if a postsynaptic depolarization is required for the induction of LTD. Therefore, two holding potentials, -70 mV and -30 mV, during 2 Hz-LFS stimulation (5 min), were tested for LTD induction. LTD is defined as a minimum 20% change of synaptic strength 15–25 min after LFS [2]. We compared averaged amplitudes of EPSCs before the LFS and 13–15 minutes and 17–20 min after the LFS. The LFS with the $V_H$ of -30 mV-induced LTD more effectively than that with the $V_H$ of -70 mV (Fig. 1). In LFS with $V_H$ of -30 mV, the mean amplitude of EPSC at baseline was 96.3 ± 3.6% (mean ± SEM) (n=7). After LFS, the mean amplitudes of EPSC were reduced to 66.8 ± 11.0% from baseline at 13–15 minutes (n=7, $P < 0.01$ vs. baseline; Fig. 3) and to 63.3 ± 9.1% from baseline at 17–20 minutes (n=7, $P < 0.01$ vs. baseline; Fig. 3). However, in the LFS with the $V_H$ of -70 mV, the mean amplitudes of EPSC were 98.0 ± 3.6% (mean ± SEM) at baseline recording, 111.4 ± 10.1% from baseline at 13–15 minutes ($P > 0.05$ vs. baseline, n=9) and 97.3 ± 6.3% from baseline at 17–20 minutes ($P > 0.05$ vs. baseline, n=9). Therefore, the initial

depression of the mean EPSC amplitudes in LFS with the $V_H$ of -70 mV was recovered within 5 minutes to the baseline level, but that upon the LFS with the $V_H$ of -30 mV was maintained, thereby, indicating the induction of LTD.

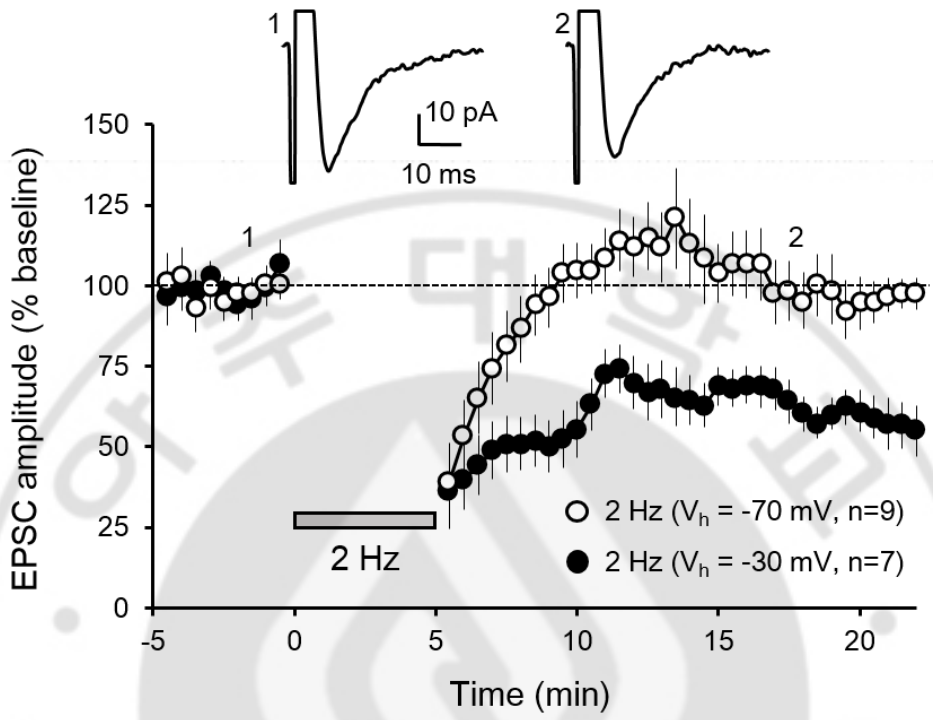


**Figure 1**. Induction of long-term depression (LTD) by low-frequency stimulation (LFS) in the spinal dorsal horn. The LTD by LFS (5 min) was induced at the $V_H$ of -30 mV (n=9, filled circle), but not -70 mV (n=7, open circle), during the conditioning stimulation. Excitatory postsynaptic currents (EPSCs) were evoked by electrical stimulation of the Lissauer's tract in the dorsal horn, particularly lamina II. 2 Hz stimulation at $V_H$ of -30 mV caused depression of the EPSC amplitude over 20 min. On the contrary, 2 Hz stimulation at $V_H$ of -70 mV produced an initial depression of EPSC amplitude, but, at 13 - 15 min, the depression returned to baseline.

## 2. OX1 and OX2 receptors mediate LTD

In the presence of 1 μM SB674042, an OX1 receptor antagonist, the depression of mean EPSC amplitudes was recovered at 13 - 15 min after stimulation with 2 Hz (5 min at the $V_H$ of -30 mV during 2 Hz; Fig. 2). The mean amplitude of EPSCs was 95.6 ± 3.7% from baseline at 13–15 min (n=8, $P > 0.05$ vs. baseline). Similarly, the mean amplitude of EPSCs was 92.7 ± 4.5% of baseline at 17–20 min (n=8, $P > 0.05$ vs. baseline) (Figs. 2 and 3). The comparison of mean EPSC amplitudes between SB674042 and control (i.e., without the drug) groups ($V_H$ of -30 mV) at both time periods revealed a significant difference ($P < 0.05$ at 13–15 min and $P < 0.05$ at 17–20 min vs. control) (Fig. 3). Therefore, the presence of 1 μM SB674042 blocked LTD after 2 Hz stimulation (5 min, the $V_H$ of -30 mV), indicating that the OX1 receptor is association with LTD development after LFS.

Furthermore, to test whether the OX2 receptor is involved in LTD development in the dorsal horn of the spinal cord, 2 Hz-LFS (5 min at the $V_H$ of -30 mV) was conducted in the presence of 1 μM EMPA in the bath solution. The depression induced after LFS was partially recovered at 13–15 min; i.e., the mean EPSC amplitude was reduced to 80.2 ± 6.8% from baseline at 13–15 min (n=7, $P < 0.05$ vs. baseline; Fig. 2). The decrease of mean EPSC amplitude did not recover sufficiently to reach a significance compared to the control group ($P > 0.05$; Fig. 3). At 17–20 min, however, the mean EPSC amplitude was recovered to 91.0 ± 6.8% of baseline (n=7, $P > 0.05$ vs. baseline; Fig.2), which was significantly different from the level of depression for the control ($P < 0.05$ vs. control; Fig.3). Therefore, the application of 1 μM EMPA in bath solution resulted in a slow recovery in the depression of EPSC amplitudes after 2-Hz stimulation (5 min at the $V_H$ of -

30 mV).

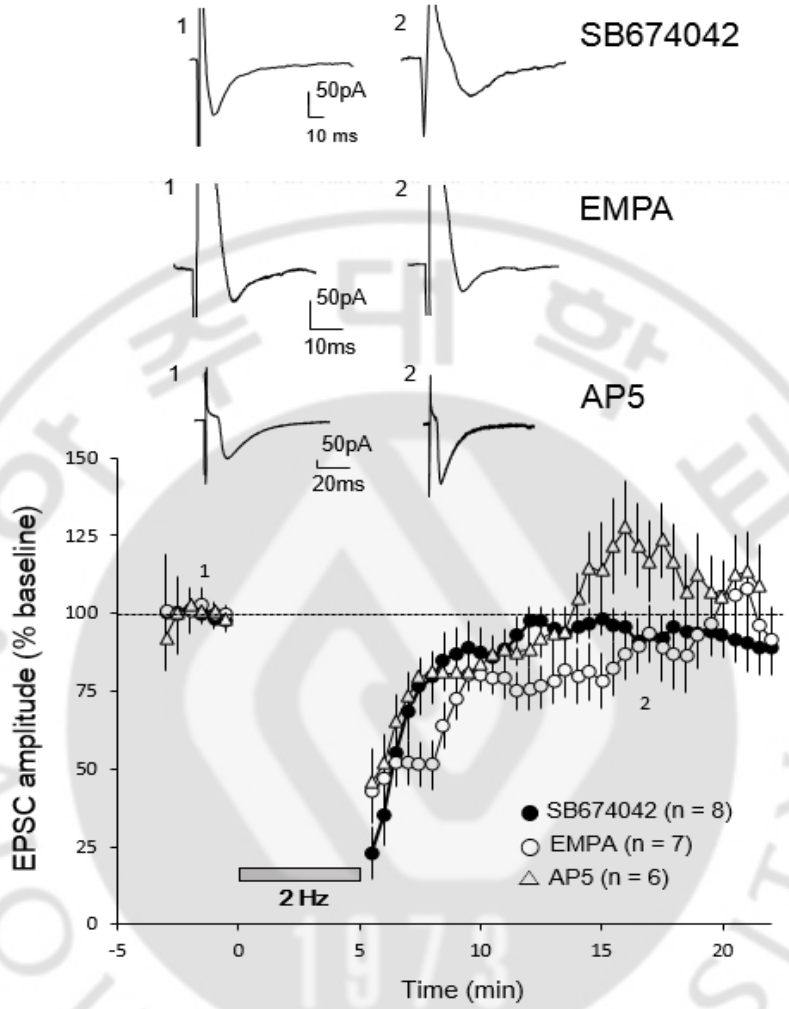


**Figure 2**. No induction of LTD in the presence of OX1, OX2 or NMDA receptor antagonists. In the presence of 1 μM SB674042, an OX1 antagonist (n=8) or 1 μM EMPA, OX2 antagonist (n=7), LTD by 2 Hz stimulation of $V_H$ of -30 mV was prevented. The depression of EPSC amplitude at the initial stage was partially recovered at 13 – 15 minutes, compared to the baseline period, and then recovered nearly to the baseline at 17-20 minutes. In the presence of 50 μM D-AP5, the NMDA antagonist (n=6), the mean EPSC

amplitude at 13 – 15 minutes did not differ from the baseline, and that at 17-20 minutest was slightly potentiated.

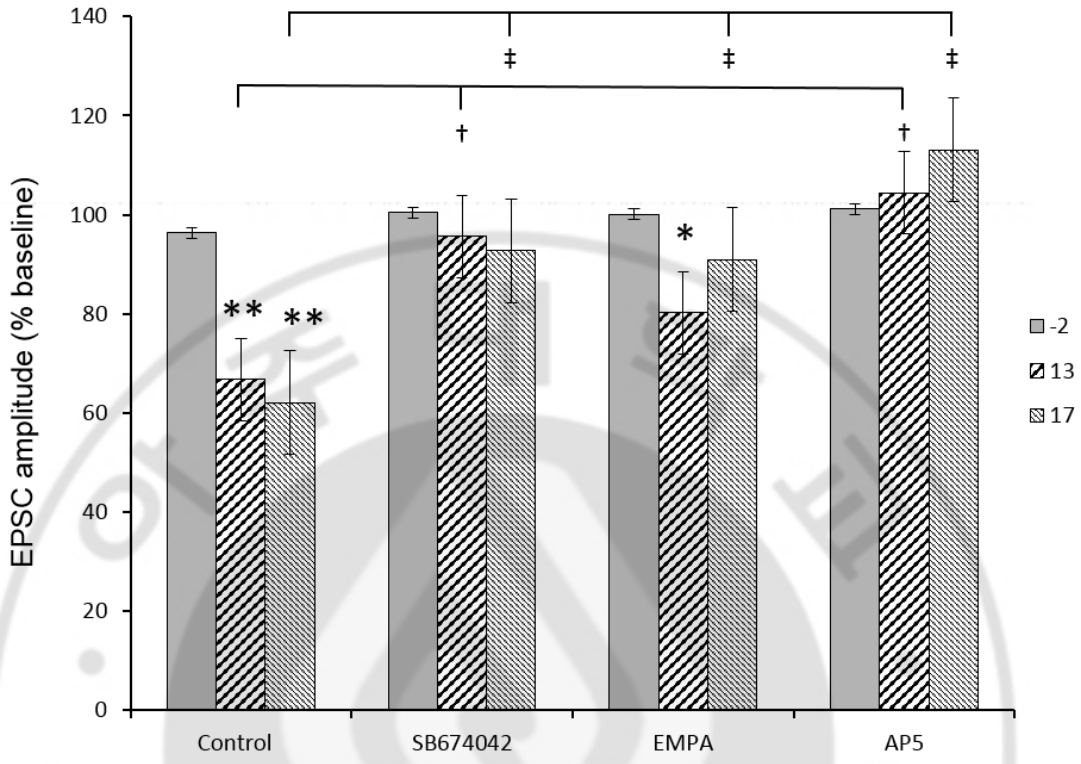


**Figure 3**. Comparison of magnitudes of LTD in different conditions. At 13-16 minutes, the mean amplitudes of EPSCs (% baseline) in SB674042 and D-AP5 were significantly different from those in the control (†$P < 0.05$). Comparisons at 17 – 20 minutes revealed differences among SB674042, EMPA and D-AP5 (‡$P < 0.05$), indicating no induction of LTD in these conditions. For bars, gray bar (-2) indicates averaged amplitude of EPSC during baseline, and bars for 13 and 17 indicate during 13-15 and 17-20 minutes, respectively. *$P < 0.05$ and **$P < 0.01$; compared with baseline within group.

## 3. LFS-induced LTD is dependent on the NMDA receptor

To test whether LFS-induced LTD is associated with the NMDA receptor, 2 Hz stimulation (5 min at the $V_H$ of -30 mV) was given in presence of 50 μM D-AP5, an NMDA receptor antagonist. In presence of 50 μM D-AP5 in the bath solution, the mean EPSC amplitudes were 104.4 ± 7.7% from baseline at 13–15 min (n=6, $P > 0.05$ vs. baseline) and, at 17–20 min, was slightly potentiated to 113.1 ± 8.3% of baseline (n=6, $P > 0.05$; Figs. 2 and 3). In comparison at both time points between control and D-AP5 groups, the mean EPSC amplitudes were significantly recovered ($P < 0.01$; Fig. 3), indicating that the NMDA receptor mediates LFS-induced LTD.

# Ⅳ. Discussion

We tested with SB674042, an OX1 receptor antagonist, and EMPA, an OX2 receptor antagonist, whether orexin receptors are involved in the expression of LTP in the spinal dorsal horn. In the presence of SB674042 at a concentration of 1 μM, the depression of the EPSC amplitude by a conditioning repetitive stimulation of 2 Hz (5 min at the $V_H$ of -30 mV) returned to the baseline control at 13–15 minutes from the initiation of conditioning. In contrast, the depression of the EPSC amplitude was partially recovered in the presence of EMPA at the concentration of 1 μM at 13–15 minutes from the initiation of conditioning. Therefore, the LTD was involved in the activation of both OX1, to a greater degree, and OX2 receptors. Further, the prevention of LTD by D-AP5 (50 μM), an NMDA receptor antagonist, indicates that this 2-Hz stimulation-induced LTD in the spinal dorsal horn is dependent on the activation of NMDA receptors.

In this study, we tested two protocols, consisting of the repetitive dorsal root stimulation at 2 Hz for 5 min combined with postsynaptic depolarizations ($V_H$ of -30 mV or -70 mV), to induce LTD. The conditioning protocol with $V_H$ of -30 mV showed LTD over a period of at least 20 min, whereas the protocol with $V_H$ of -70 mV revealed short-lasting depression for less than 5 min. Therefore, the protocol with $V_H$ of -30 mV was used in this study. Besides, we stimulated the Lissauer's tract in the spinal cord slices to evoked EPSCs. The Lissauer's tract is a pathway formed from the proximal end of small unmyelinated and poorly myelinated primary afferent fibers which enter the lateral aspect of the dorsal horn. Fibers from the Lissauer's tract principally extend to laminae I/II and V of

the ipsilateral dorsal horn. Consequently, the stimulation to Lissauer's tract zone with low frequency (2Hz) may activate orexinergic fibers coming from the primary afferent fiber. On the other hand, lamina I is densely innervated by long descending axons from orexin-positive neurons in the hypothalamic area. Thus, the stimulation of Lissauer's tract may activate, in some way, the descending orexinergic fibers. Therefore, during the repetitive low-frequency stimulation, orexins release from both primary afferent and descending terminals, contributing LTD in the spinal dorsal horn [14].

Orexins are derived from prepro-orexins, which can be detected in neurons of the lateral and posterior hypothalamus [7]. The neurons synthesizing orexins send their axonal projections to various areas of the brain, and control arousal, sleep, and food intake behaviors [7,15]. Orexinergic terminals are also distributed in the superficial dorsal horn of the spinal cord, and participate in the modulation of nociceptive inputs from primary afferent fibers [14]. Intrathecally-administrated orexin A attenuates the mechanical allodynia induced by sciatic nerve ligation [10], the effect being mediated by orexin receptors in the spinal cord. Furthermore, it was revealed that orexin A, but not orexin B, is effective to reduce hyperalgesia in chronic constriction injury of sciatic nerve [16]. Our results are in line with these findings. SB674042 prevented the depression of EPSCs after the conditioning stimulation at 13–15 min, as did EMPA partially at 13–15 min and comprehensively at 17–20 min. In a view that LTD may be a cellular substrate of antinociceptive effects in the spinal dorsal horn [17], our study suggests that OX1 and OX2 receptor-mediated LTD reflects the attenuation of nociceptive behaviors by the intrathecal injection of orexins.

Orexin A increased calcium influx in dorsal root ganglion and spinal dorsal horn neurons through OX1 receptor, which supports its modulating

role on nociception [18]. Orexin receptor is G-protein coupled receptor (GPCR), and the stimulation of orexin receptor leads to activation of phospholipase C (PLC) and subsequent activation of Protein kinase C (PKC) [19]. In the ventral tegmental area (VTA), it was known that orexin A increases calcium influx through the PLC-PKC process. PLC or PKC inhibitors abolished calcium increase in VTA after the application of orexin A, additionally support the mediation of PLC and PKC for orexins' effect [20]. Similar as this finding, PKC inhibitor abolished intracellular calcium increase in dorsal root ganglion neurons [21], suggesting that the PLC-PKC-calcium rise pathway mediates physiological functions of orexin.

LTP and LTD were affected by many postsynaptic receptors. The upregulation of the AMPA receptor is critical for the expression of LTP in the CA1 of the hippocampus, and its downregulation for LTD in the cerebellum [22]. Moreover, the activation of the NMDA and AMPA receptors play a prominent role in the induction of LTP in the spinal dorsal horn [2,23]. The induction of LTD requires moderate calcium influx; in contrast, LTP needs high calcium elevation [24]. In learning and memory, LTD is associated with memory loss; however, LTP is associated with memory enhancement [25]. In the spinal cord, LTP has been proposed as the central sensitization, a mechanism of hyperalgesia. Brief tetanic stimulation of the sciatic nerve at the Aδ fiber strength reduces the amplitude of C-fiber-evoked field potential, inducing LTD [26]. LTD was blocked or strongly reduced by the NMDA antagonist D-AP5 or the $Ca^{2+}$ chelator BAPTA [3]; indicating that this type of LTD is NMDA receptor-dependent [27]. In our experiments, D-AP5 prevented the depression of EPSCs amplitudes after the conditioning stimulation, suggesting that our protocol evoked NMDA receptor-dependent LTD in the spinal dorsal horn, similarly to other studies [3]. Further, the prevention of LTD by OX1 or OX2 antagonists suggests

that orexins involve NMDA receptor-dependent LTD in the spinal dorsal horn.

From a clinical perspective, LTD modifies peripheral inputs coming into the spinal dorsal horn, indicating an analgesic effect in the nociceptive pathway. In practice, repetitive transcranial magnetic stimulation (rTMS) has been applied for the treatment of epilepsy. Low-frequency rTMS reverses LTP in epilepsy as a result of reducing circuit excitability [28]. In humans, an application of a similar protocol, which evoked LTP or LTD in spinal cord slices, induced hyperalgesia or hypoalgesia compared to the control condition [6]. In light of the mediation of orexin receptors in the development of LTD, orexins may contribute to pain reduction in acute pain conditions by reducing central sensitization, i.e., the counter-action of LTP.

# Ⅴ. Conclusion

In the present study, we observed that LTD was evoked by low frequency stimulation at 2 Hz for 5 min with $V_H$ of -30 mV. The LTD was mediated by OX1, OX2, or NMDA receptors in its development. This may represent a mechanism that orexins exert analgesic effects in nociceptive or neuropathic pain models.

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-국문요약-

신경펩티드 오렉신 A와 B는 수면 및 각성과 섭식 행동을 조절한다. 최근 이 펩티드는 여러가지 통증 모델에서 항통각 효과가 있음이 밝혀졌다. 하지만 오렉신이 시냅스의 효과를 각각 증가시키거나 감소시키는 장기 증강 (long-term potentiation) 과 장기 억압(long-term depression)과 같은 시냅스 가소성(synaptic plasticity)에 어떻게 관여하는지는 분명하지 않다. 이 연구에서, 저자들은 오렉신 수용체 1형과 2형이 어린 래트의 척수 횡절편에서 흥분성 시냅스 전달의 유발과 유지에 관여하는지 알아보았다.

리사웨르로 존 (Lissauer's tract zone)에 전기 자극을 2 헤르츠(Hz) 5분 동안 (600 pulses) 고정 전위 -30mV로 반복적으로 가하면 일차 구심성 섬유의 활성화에 의해 유발되는 흥분성 연접 후 전류(excitatory postsynaptic currents)의 진폭(amplitude)에 장기 억압을 유발하였다. 장기 억압의 유지는 오렉신 수용체 1형의 길항제인 SB674042(1 μM) 혹은 2형의 길항제인 EMPA (1 μM)을 배스에 적용하여 의미 있게 차단되었다. 이에 더하여 장기 억압은 NMDA 수용체 길항제인 D-AP5로 차단되는 것으로 보아 NMDA 수용체에 의존적이다. 우리 연구는 오렉신 수용체 1,2형을 통해 오렉신이 NMDA 의존적 장기 억압의 발현에 중요한 역할을 함으로써 통증 전달에서 척수 조절에 기여하는 것으로 보인다.