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# Effects of [D-Pen$^2$, D-Pen$^5$]-enkephalin on the Neuronal Activity of Medial Vestibular Nuclear Neurons

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This study was designed to investigate direct effects of [D-Pen$^2$, D-Pen$^5$]-enkephalin, a δ-opioid receptor agonist on the neuronal activity of medial vestibular nuclear (MVN) neurons by whole-cell configuration patch clamp experiments. The spike frequency of MVN neuron was increased to 9.50±0.55 ($P$<0.05) and 10.56±0.66 ($P$<0.05) by 5 and 10 μM [D-Pen$^2$, D-Pen$^5$]-enkephalin from the control level of 8.05±0.55 spikes/sec, respectively (n=18). The resting membrane potential of the neurons was increased to -37.86±0.92 and -36.97±0.97 ($P$<0.05) from -38.74±1.13 mV by 5 and 10 μM [D-Pen$^2$, D-Pen$^5$]-enkephalin, respectively. The amplitude of afterhyperpolarization was decreased to 23.78±0.65 and 21.67±0.89 ($P$<0.05) from 23.73±0.53 mV by 5 and 10 μM [D-Pen$^2$, D-Pen$^5$]-enkephalin, respectively. The spike width was changed to 2.22±0.08 and 2.24±0.07 from 2.20±0.08 mV by 5 and 10 μM [D-Pen$^2$, D-Pen$^5$]-enkephalin, respectively. After pretreatment of naltrindole, a highly selective δ-opioid receptor antagonist, [D-Pen$^2$, D-Pen$^5$]-enkephalin did not change firing rate, resting membrane potential, afterhyperpolarization amplitude, and spike width of MVN neurons. The above experimental results suggest that [D-Pen$^2$, D-Pen$^5$]-enkephalin increases the neuronal activity of MVN neurons via inhibition of calcium-dependent potassium currents underlying the afterhyperpolarization.

**Key Words:** Action potential, [D-Pen$^2$, D-Pen$^5$]-enkephalin, Opioid, Vestibular nuclear neuron

## INTRODUCTION

Numerous morphological and functional studies have been done to demonstrate the presence of enkephalinergic neurons and its action in vestibular nuclei (Lin and Carpenter, 1994; Sulaiman and Dutia, 1998; Zanni et al., 1995; Beitz et al., 1987; Nomura et al., 1984). Although several lines of evidence suggested that opioid can play a possible role as a neurotransmitter or neuromodulator in the central vestibular system, its action on the activity of vestibular nuclei is not reported to be consistent. Lin and Carpenter reported that opioid activates the medial vestibular nuclear (MVN) neurons (Lin and Carpenter, 1994). But Sulaiman and Duita demonstrated inhibitory effects of delta-opioid receptors on the medial vestibular nuclear neurons (Sulaiman and Dutia, 1998).

Vestibular compensation is a process of gradual functional recovery from vestibular deficeits such as spontaneous nystagmus, unilateral head tilt and timing and gain abnormality of vestibular reflexes after unilateral labyrinthectomy (UL) (Aldrich and Peusner, 2002; Precht and Dieringer, 1985). Not only the effects of opioid on vestibular neuronal activity but also the effects of opioid on the vestibular compensation are controvertial. Systemic administration of naloxone, an opioid antagonist enhances ocular motor compensation in guinea pig after vestibular deafferentation (Dutia et al., 1996). By contrast, Saika et al. reported that decay profile of preproenkephaline mRNA expression up-regulated in ipsilateral medial vestibular nuclear neurons after unilateral labyrinthectomy is close related to the vestibular symptoms (Saika et al., 1993).

It is reported that rebalancing of neuronal activity of ipsilateral and contralateral vestibular nuclei is important for vestibular compensation after UL (Ris and Godaux, 1988; Darlington and Smith, 1996). Various neurotrans-

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mitters are involved in the rebalancing of neuronal activity. It is meaningful to identify the functions of these neurotransmitters for understanding its role in vestibular compensation. This study was designed to investigate direct effects of [D-Pen$^2$, D-Pen$^5$]-enkephalin, a δ-opioid receptor agonist and action mechanisms on MVN neurons. We performed whole-cell configuration patch clamp experiments under a current-clamp mode, on acutely isolated rat MVN neurons.

## METHODS

### 1. Animals and preparation of medial vestibular nuclear neurons

Institutional Committee of Laboratory Animal Care and Use approved the experimental protocol. Coronal slices of the brainstem of Sprague-Dawley rats aged 14 to 17 days were prepared as described previously for rats (Kay and Wong, 1986). Briefly, the animals were anesthetized with ether and decapitated. The brainstem was rapidly removed into ice-cold artificial cerebrospinal fluid.

The coronal slices (400-μm-thick) of the brainstem were made with a sliding microtome (Vibroslice; WPI, Sarasota FL, USA). These slices were incubated in artificial cerebrospinal fluid well saturated with 95% $O_2$ / 5% $CO_2$ at room temperature for 1 h. The slices were treated with pronase (0.2 mg/ml) for 40~60 min and subsequently exposed to thermolysin (0.2 mg/ml) for 10 min at 32℃. After the enzyme digestion, a portion of MVN neurons was removed by micropunching and gently agitated. The dissociated neurons were transferred into a recording chamber mounted on an inverted microscope (IX 70; Olympus, Tokyo, Japan).

### 2. Whole-cell patch-clamp

The whole-cell membrane potentials were recorded at room temperature by using standard patch-clamp techniques (Hamill et al., 1981). The patch pipette had a resistance of 3~6 MΩ when filled with a pipette solution. Membrane potentials were measured with an Axopatch 200B voltage-clamp amplifier (Axon instrument, Foster City, CA, USA). Command pulses were applied using an IBM-compatible computer and pCLAMP 7 software (Axon instrument, Foster City, CA, USA). The data were filtered at 5 kHz and displayed on an oscilloscope (Tektronik, Wilsonville, OR, USA), a computer monitor, and a pen recorder (Polygraph; Grass, Quincy, MA, USA).

### 3. Internal and external solutions

The artificial cerebrospinal fluid had the following composition (mM): NaCl 124, KCl 5, $KH_2PO_4$ 1.2, $MgSO_4$ 1.3, $CaCl_2$ 2.4, D-Glucose 10, $NaHCO_3$ 24. The external solution for recordings had the following composition in mM: NaCl 124, KCl 5, $MgSO_4$ 1.3, $NaHCO_3$ 26, $CaCl_2$ 2, $NaH_2PO_4$ 1, Glucose 11 (pH 7.4 with KOH). The internal solution (the patch pipette solution) had the following composition in mM: K-gluconate 122.5, KCl 17.5, NaCl 8,

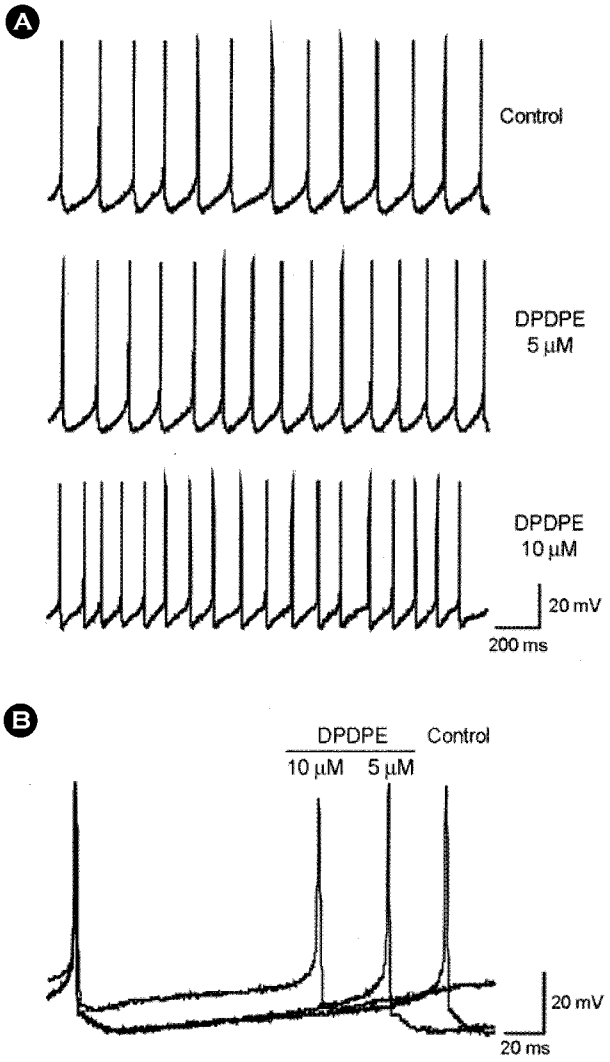


**Fig. 1.** Effects of [D-Pen$^2$, D-Pen$^5$]-enkephalin (DPDPE) on spontaneous action potentials of medial vestibular nuclear neurons. **(A)** Control, 5 and 10 μM DPDPE; **(B)** averaged spike shapes.

HEPES 10, EGTA 0.5, Mg-ATP 4 (pH 7.3 with KOH).

### 4. Drugs

The drugs were made from stock solutions that were made up in distilled water and diluted to the desired concentration in external solution. The drugs were applied to the MVN neurons by switching the perfusion inlet tube to the bath chamber. They were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Data of the same drug tested in this study was obtained in the single neuron with sequential administration of different concentrations. The average firing rate and membrane potential were calculated in recordings over 10 minutes. The resting membrane potential was measured at the lowest point of the rising phase of the spike. The afterhyperpolarization amplitude of the action potential was measured as the difference of membrane potential between the spike threshold and the minimum post-falling phase of the spike.

### 5. Statistics

All values are expressed as mean ± S.E.M. The one-way ANOVA test (Bonferroni Post Hoc comparison) was used to analyse the differences between groups, with $P<0.05$ being considered significant.

## RESULTS

The whole-cell patch clamp recordings under the current-clamp mode were performed to investigate the direct effects of [D-Pen$^2$, D-Pen$^5$]-enkephalin, a δ-opioid receptor agonist

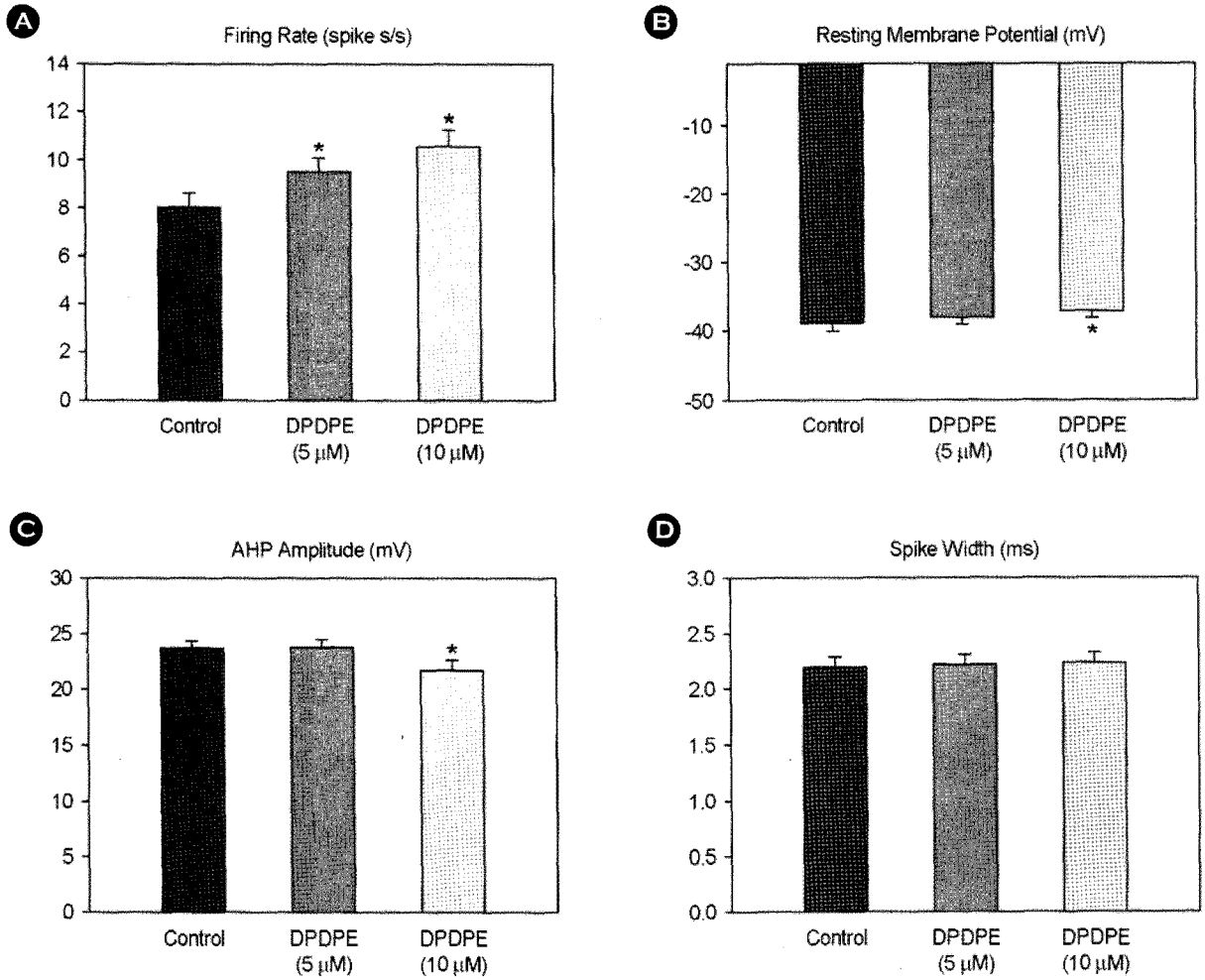


**Fig. 2.** Effects of [D-Pen$^2$, D-Pen$^5$]-enkephalin (DPDPE) on firing rate **(A)**, resting membrane potential **(B)**, afterhyperpolarization (AHP) amplitude **(C)**, and spike width **(D)**, of medial vestibular nuclear neurons (n=18, *significantly different from the control with $P<0.05$).

on the spontaneous activity of the MVN neurons. When the command current was fixed to 0 nA, the neurons revealed spontaneous firing action potentials with a frequency of 7.53±0.33 spikes/sec.

All of the 18 cells responding to [D-Pen$^2$, D-Pen$^5$]-enkephalin showed excitatory responses under current-clamp mode. The spike frequency of MVN action potential was increased to 9.50±0.55 ($P$<0.05) and 10.56±0.66 ($P$< 0.05) by 5 and 10 μM [D-Pen$^2$, D-Pen$^5$]-enkephalin from the control level of 8.05±0.55 spikes/sec, respectively. The resting membrane potential of the neurons was increased to -37.86±0.92 and -36.97±0.97 ($P$<0.05) from -38.74± 1.13 mV by 5 and 10 μM [D-Pen$^2$, D-Pen$^5$]-enkephalin, respectively. The amplitude of afterhyperpolarization was decreased to 23.78±0.65 and 21.67±0.89 ($P$<0.05) from 23.73±0.53 mV by 5 and 10 μM [D-Pen$^2$, D-Pen$^5$]-enkephalin, respectively. The spike width was changed to 2.22±0.08 and 2.24±0.07 from 2.20±0.08 mV by 5 and 10 μM [D-Pen$^2$, D-Pen$^5$]-enkephalin, respectively (Figs. 1 and 2).

The effects of [D-Pen$^2$, D-Pen$^5$]-enkephalin on the MVN neurons were antagonized by pretreatment of naltrindole, a highly selective δ-opioid receptor antagonist. After pretreatment of naltrindole, [D-Pen$^2$, D-Pen$^5$]-enkephalin did not change firing rate, resting membrane potential, afterhyperpolarization amplitude, and spike width of MVN neurons (Figs. 3 and 4).

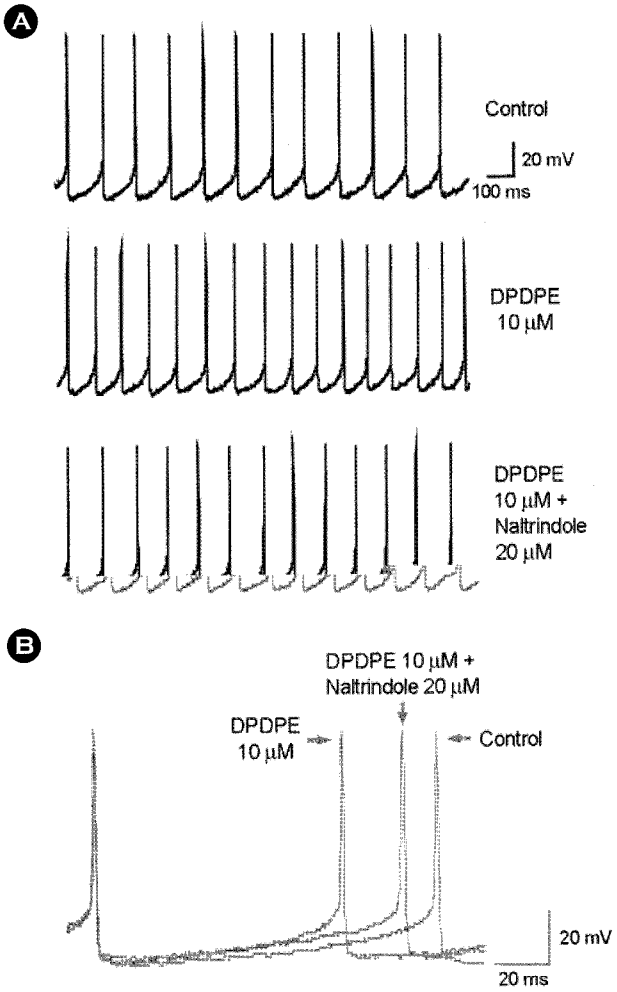


**Fig. 3.** Blocking effects of naltrindole, a highly selective δ-opioid receptor antagonist on the action of [D-Pen$^2$, D-Pen$^5$]-enkephalin (DPDPE). **(A)** Control, 10 μM DPDPE, and combination of 10 μM DPDPE and 20 μM naltrindole; **(B)** averaged spike shapes.

## DISCUSSION

Studies on the control of neuronal activity by opioids have reported diverse effects, predominantly inhibitory by direct postsynaptic hyperpolarization or decreasing the release of neurotransmitters through activation of presynaptic receptors (North and Williams, 1985; Pan et al., 1990). Sulaiman and Dutia who performed extracellular and whole-cell patch clamp intracellular recordings on MVN reported that δ-opioid receptor agonists inhibited MVN neurons, but μ, κ-opioid receptor agonists did not affect the tonic discharge rate of neurons (Sulaiman and Dutia, 1998).

By contrast, Yasnetsov and Pravdivtsev reported iontophoretic application of opioid agonists, morphine or methionine and leucine-enkephaline increased neuronal firing of MVN (Yasnetsov and Pravdivtsev, 1986). Lin and Carpenter also reported majority of MVN neurons were excited by ionophoretically applied μ and δ-opioid receptor agonists, morphine and [D-Ala$^2$]-leucine-enkephalin, using extracellular recordings (Lin and Carpenter, 1994). They interpreted that the excitatory effects of opioids result from direct membrane depolarization of MVN neurons rather than disinhibition. The experimental results of our study are consistent with those of Lin and Carpenter using extracellular recordings. It is difficult to demonstrate the reasons why the experimental results are different to each other. Further extensive studies on the function of opioids in MVN are required with various experimental methods.

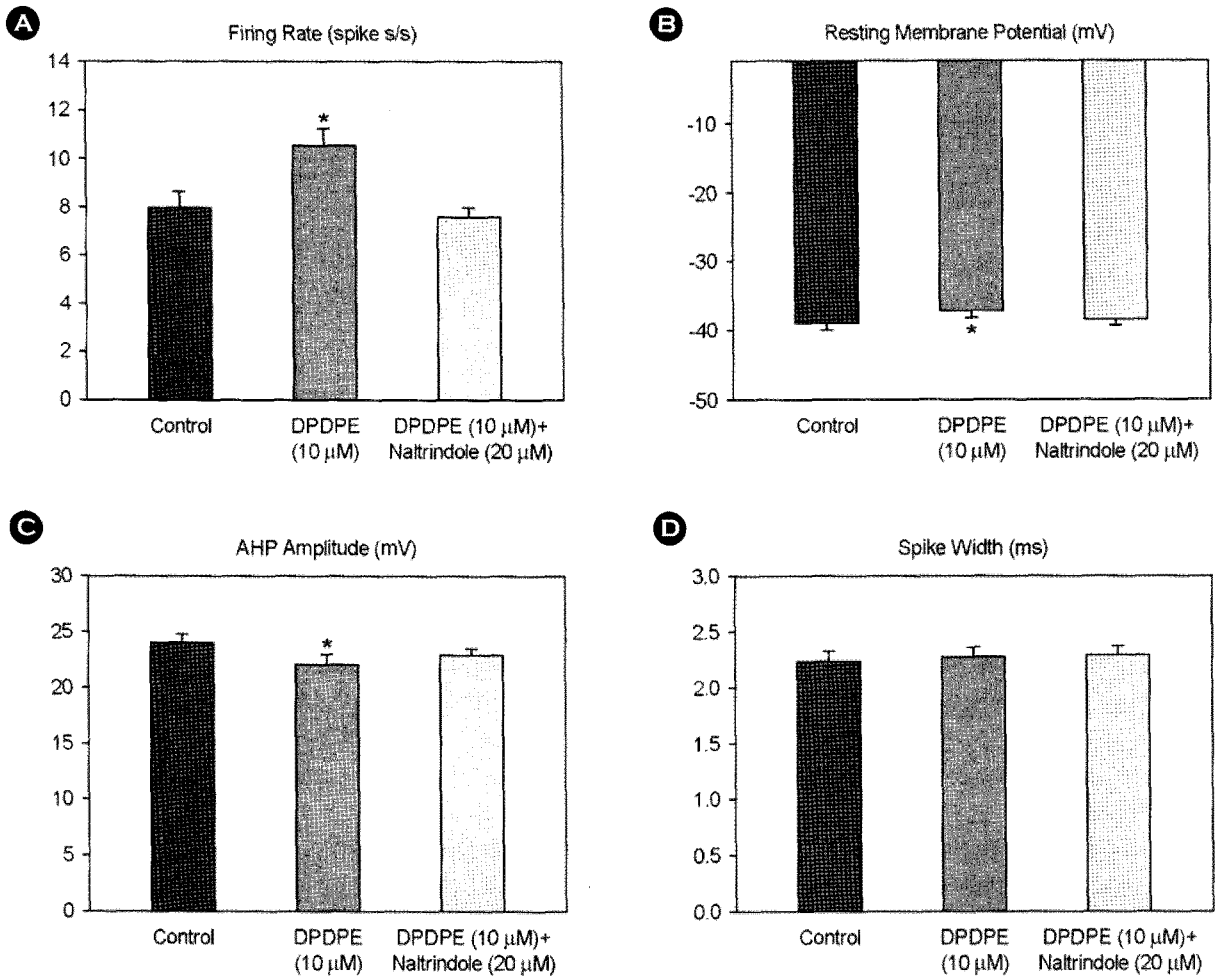


**Fig. 4.** Effects of naltrindole on the firing rate **(A)**, resting membrane potential **(B)**, afterhyperpolarization (AHP) amplitude **(C)**, and spike width **(D)**, of medial vestibular nuclear neurons (n=18, *significantly different from the control with $P<0.05$).

Duita et al. reported that systemic administration of naloxone reduced the frequency of spontaneous nystagmus after UL, while it did not improve the postural symptoms such as yaw head tilt and roll head tilt significantly (Dutia et al., 1996). They suggested the effects of systematically administrated naloxone are induced by acting directly on the MVN, not by acting on other areas of the CNS. And they also suggested the beneficial effects of naloxone are produced by reducing the intracellular calcium concentration in MVN neurons.

Recently, Kitahara et al. reported an interesting study concerned with up-regulation in preproenkephalin (PPE)-like immunoreactivity (-LIR), in the ipsilateral MVN after UL (Kitahara et al., 2006). They observed substantial number of ipsilateral MVN neurons revealed both PPE-LIR and Fos-LIR, a marker of neuronal activation by 6 hour with a maximum increase in number 1 day after UL. The time decay of the double stained neurons was parallel with that of functional deficits induced by UL. Contrary to the study of Duita et al., PPE antisense and naloxone studies revealed that depression of enkephalinergic effects in ipsilateral MVN delayed the vestibular compensation, especially at the chronic stage.

Kitahara et al. suggested a hypothesis on the role of Fos-PPE signaling in vestibular compensation. After UL, Fos is induced and Fos protein regulates PPE and enkephalin expression in ipsilateral MVN. After synthesized in the interneurons of MVN, opioids participate in restoration of the neuronal activity of type I MVN neurons. Because effects of opioids on the neuronal activity of MVN are different based on two previous reports, they suggested two ways for opioids to participate in enhancing the decreased

neuronal activity of ipsilateral MVN, direct activation of type I neurons or indirect activation via inhibition of the inhibitory interneurons which acting on type I neurons.

In the present study, functional roles of δ-opioid receptor on MVN neurons were explored by whole-cell configuration patch-clamp experiments. [D-Pen$^2$, D-Pen$^5$]-enkephalin, a δ-opioid agonist increased the spontaneous firing rate, decreased the resting membrane potential and the amplitude of afterhyperpolarization. Taken together with our experimental results and those of Kitahara et al., opioid might facilitate the functional recovery after UL by increasing the neuronal activity via δ-opioid receptors on type I MVN neurons. Neuronal activity can be modulated by changes of resting membrane potential or afterhyperpolarization in MVN neurons. In our previous reports, CGS-12066A, a selective 5-HT1B receptor agonist and EGCG decreases the spontaneous firing rate of MVN neurons (Jeong et al., 2005). By contrast, 5-a-methylhydroxytryptamine, a 5-HT2 receptor agonist increases the firing rate of MVN neurons by depolarizing the neurons, which is similar to the action of opioid in the present study (Jeong et al., 2003).

[D-Pen$^2$, D-Pen$^5$]-enkephalin changes not only the resting membrane potential but also the afterhyperpolarization in the present study. Afterhyperpolarization is known to be underlied by calcium-dependent potassium currents (Peusner et al., 1998). Although it is not tested whether [D-Pen$^2$, D-Pen$^5$]-enkephalin affects the calcium-dependent potassium currents, [D-Pen$^2$, D-Pen$^5$]-enkephalin could decrease the calcium-dependent potassium currents in MVN neurons. In the previous study, opioids are reported to modulate the voltage-dependent and calcium-dependent potassium currents in cultured bovine adrenal medullary chromaffin cells (Twitchell and Rane, 1994). Further studies are required to explore the action mechanisms of opioids in MVN neurons using voltage-clamp experiments and calcium imaging studies.

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