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The effect of KCNQ4 activator on acute vestibular dysfunction in mouse

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The effect of KCNQ4 activator on acute vestibular dysfunction in mouse

Directed by Professor Sunghuhn Kim

The Master's Thesis
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

Kangyoon Park

December 2023

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This certifies that the Master's Thesis of
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ABSTRACT

The effect of KCNQ4 activator on acute vestibular dysfunction in mouse

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The Graduate School, Yonsei University

(Directed by Professor Sunghuhn Kim)

Acute unilateral vestibulopathy induces vertigo, spontaneous nystagmus, and postural instability. So far, there is no treatment method to recover the vestibule function, instead, visual and postural stability accomplished by central vestibular compensation. KCNQ4 is a voltage-gated K^+ channel distributed over afferent nerve of vestibular hair cells. The role of KCNQ4 in the vestibular system is not fully understood. We tried to investigate the role of KCNQ4 in the acute vestibular loss in mouse model. Labyrinthectomy was performed in C57BL/6 mouse with the intraperitoneal application of retigabine (a KCNQ4 activator) or DMSO immediate and 12 hours after the surgery. Vestibular function changes after the labyrinthectomy in each mouse group was examined by measuring the average slow velocity of spontaneous nystagmus and vestibulo-ocular reflex(VOR) in slow harmonic acceleration (SHA). They were measured before the surgery, immediately after the surgery, 1, 12, 48 hours, and 7 days after the injection of each reagent. The same tests were also performed at *Kcnq4*$^{p.W277S/p.W277S}$ mouse to confirm the effect of retigabine on KCNQ4. The average slow phase velocity of spontaneous nystagmus after the surgery decreased with time in both groups, but the average velocity was significantly decreased more in the mice with retigabine injection. There was no significant difference in SHA test gain until 48 hours after the injection between both groups. However, the retigabine-injected group showed significantly increased gain at 1.28Hz on 7 days after the injection. In contrast, there was no statistically significant difference in the parameters of VOR

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measurement in the same experiment using *Kcnq4*$^{p.W277S/p.W277S}$ mouse. To investigate the role of KCNQ4 in the central vestibular system, immunohistochemistry of c-fos in medial vestibular nucleus (MVE) was performed and the expression of c-fos and KCNQ4 was more increased in retigabine-injected mice when compared to DMSO-injected group. The findings suggested that vestibular compensation could be induced more rapidly by the stimulation of KCNQ4 in the central vestibular system.

Key words : vestibular dysfunction, unilateral labyrinthectomy, retigabine, KCNQ4, Kv7.4, Vestibulo-Ocular Reflex, Central vestibular compensation, brain stem, medial vestibular nucleus, C-Fos

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The effect of KCNQ4 activator on acute vestibular dysfunction in mouse

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I. INTRODUCTION

The vestibular organs of the inner ear contribute to maintain the balance of the body and stability of vision during movement by detecting acceleration stimulation and gravity changes.[1] The acceleration stimulation induces hair cell cilia tilt in the vestibular organ and K^+ ions of the endolymph flux into the hair cell through the mechano-sensitive non-selective cation channel at the cilia.[1] The K^+ influx induces hair cell depolarization and the changes of membrane potential activate voltage-gated Ca^{2+} channels on the basolateral membrane of the hair cells, which finally induces neurotransmitter release to activate vestibular afferent nerve.[2] The K^+ ions escape from the hair cell, leading to hyperpolarization through K^+ channels on the basolateral membrane of the hair cells. KCNQ4 (Kv7.4) is revealed to be present on the inner surface of the synaptic calyx and heminode of type I hair cell and thought to contribute to the regulation of depolarization and hyperpolarization of hair cell and vestibular nerve.[3] So far, nothing has been revealed in the presence of KCNQ4 in the central vestibular system.

An acute vestibular injury, such as vestibular neuritis, causes severe vertigo and body imbalance. The cardinal findings of acute vestibular loss are represented by unidirectional spontaneous nystagmus and sway or falling to lesion side, which persists days to weeks.[4] The nystagmus and vertigo are resolved after several days to week by central vestibular compensation, but vestibular loss in the lesion side after injury mostly is not recovered.[5] So far, most treatment strategy for acute vestibular loss is focused to enhance vestibular

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compensation by vestibular rehabilitation exercise and there is no treatment method to recover the vestibular function.[6] Therefore, it would be great advance in the treatment of acute vestibular loss if there is a medical treatment to enhance vestibular compensation or functional recovery of injured vestibular organ.

In this study, we tried to investigate if the activation of KCNQ4 in the vestibular afferent system can contribute to the symptom recovery and enhancement of vestibular compensation. We applied KCNQ activator retigabine before and after the labyrinthectomized mouse model and investigate the recovery of vestibulo-ocular reflex and resolution of spontaneous nystagmus. In addition, we examine the presence of KCNQ4 and increased expression of c-fos in the vestibular nucleus to investigate if KCNQ4 contribute to enhancement of vestibular compensation. We believe that the result of this study can be a basis for understanding the vestibular recovery / compensation and the development of new treatment method for acute vestibular injury.

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II. MATERIALS AND METHODS

1. Mouse model

Eight-weeks old C57BL/6 and Kcnq4p.W277S/p.W277S mouse with severe KCNQ4 dysfunction (Topsakal, et al., 2005) was used for the experiments. A p.W277S/p.W277S Kcnq4 knock-in mouse model was generated by Macrogen Inc. (Seoul, Korea) using a CRISPR/Cas9 technique, as previously reported (Wang et al., 2016). The 830G>C mutation in exon 5 was applied to p.W277S/p.W277S Kcnq4 transgenic mice. The transgenic mouse model was established with a CRISPR/Cas9 system. Two 23 nt guide RNAs, 5'- CCTCCTATGCCGACTCGCTCTGG -3' and 5'- ATGCCGACTCGCTCTGGTGGGGG -3', were designed to drive Cas9 to the target exon 5 of the Kcnq4 gene, where mutation 830G>C is located.

Genotype sequencing was performed with

sense primer 5'- AGGCTGGAAAGGCGATG -3',

anti-sense primer 5'- CGGTACACATCACAAGGGCT -3',

restriction enzyme NdeI, and a silent mutation for preventing recut of the knock-in allele by single guide RNA (size of amplicons: wild-type: 1,291 bp, knock-in: 656 bp + 635 bp). The background of the Kcnq4p.W277S/p.W277S mice was C57BL6/J, but in the embryo transfer procedure, the mouse background was mixed with C57BL6/N (Orient Bio, Seoul, Republic of Korea). The Kcnq4+/p.W277S mice were made by cross-breeding Kcnq4+/+ (C57BL6/N) and Kcnq4p.W277S/p.W277S mice. All the experiments were conducted using 8- to 12-week-old mice.

2. Surgical procedure and pharmacological agent treatment

Inhalation narcosis by isoflurane was performed in 8-week-old mice. An 1cm long retroauricular vertical incision was made at 0.5cm from the right auricle. The soft tissue and muscles were dissected with an electrocautery to posterior semicircular

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canal and the lateral semicircular canal and trephination was made with a dental drill. The endolymph and perilymph were completely sucked out by continuous suction and seal the wound after the suction. At 1 and 2 hours after the labyrinthectomy, 10 μg/g of retigabine diluted in DMSO (experimental group) and DMSO (control group) was injected intraperitoneally.

3. VOR recording

After inhalation narcosis described above, a skin incision approximately 1 cm in length was made anteriorly from the vertex of the mouse head, and a small metal nut with a screw hole for the fixation of the head to the head holder in the animal rotator was anchored to the vertex using dental cement. We waited 2-3 days for the stabilization of the head holder. Before the experiment, the mouse was anesthetized with isoflurane gas (2-4%), and triangular paper markers (each side = 0.4 mm) for eye tracking were attached to the center of the eyeball using an adhesive. Then, the mouse was placed in a cylindrical restrainer connected to the vestibular turntable. The mouse's head was fixed to the restrainer using the previously implanted head fixation anchor in the scalp. The VOR was measured after the mouse was completely awaken from the anesthesia. The measurements were performed before surgery, immediate after surgery, at 1, 2, 12, 48 hours and 7 days after first injection. Slow harmonic acceleration (SHA) stimuli with stimulation frequencies of 0.08, 0.1, 0.16, 0.32, 0.64, and 1.28 Hz was applied. Among the VOR parameters, gain value (slow phase peak eye velocity induced by rotation divided by peak rotation velocity) was analyzed.

4. Immunohistochemistry

After 4 hours of labyrinthectomy and the injection of retigabine / DMSO, the cardiac perfusion was performed by opening the chest of the anesthetized mouse. A 28 gauge needle was inserted into the left ventricle of the mouse and inject 10ml of

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phosphate-buffered saline (PBS, 1X, pH 7.4) and 4% paraformaldehyde (PFA) sequentially. After the cardiac perfusion mouse brain stem was harvested carefully. The harvested tissue was fixed for 2 hours in PFA. Then, the fixed brain was immersed in 30% sucrose for 2-3 days for dehydration. The dehydrated brain was placed in a cryo−mold filled with O.C.T compound (Sakura Finetek, CA90501, USA). The prepared tissue was placed in cryotome and sectioned (30μm) at the vestibular nucleus level. After attaching the cut pieces to the positively charged glass slide the tissue was blocked 10% normal donkey serum (NDS, AB_2337258, Jackson ImmunoResearch, USA) in 5%BSA for 30 minute in room temperature. Then, the tissue was washed 3 times for 10 minute with 0.2% PBS-TX. c-fos rabbit polyclonal antibody (1:500, PA1-830, ThemoFisher, USA) and mouse monoclonal anti-KCNQ4 antibody (1:500, ab84820, abcam, USA) was applied for 1 hour in room temperature and the tissue was washed 3 times by 10 minute with PBS-TX. Alexa 568 and Alexa 488 secondary anti-body (1:1000), and DAPI in 0.2% PBS-TX was applied for 1 hour in room temperature. The tissue was washed 5 times with PBS-TX and covered by coverslips with Fluoromount Aqueous Mounting Medium (F4680, Sigma-Aldrich, USA). Prepared tissues were imaged using LSM 980 (Zeiss Laboratories, Germany).

5. Statistics

In each dataset, normality and equal variance were evaluated by the Shapiro-Wilk test and Brown-Forsythe test. For the comparison of average slow phase velocity difference among different time point, one-way ANOVA or one-way ANOVA on ranks was used for parametric and non-parametric data analysis. Comparison of the difference between the average slow phase velocity of spontaneous nystagmus and gain value in SHA test, t-test or Mann Whitney U test was used for parametric and non-parametric data analysis. $p < 0.05$ was considered to be significant. Statistical analysis was performed with SigmaPlot 13.0 (Systat Software, San Jose, CA, USA

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6. **Study approval**

All the animal experimental procedures and a study enrolling humans were approved by the Yonsei University Health System Institutional Animal Care and Use Committee (approval No. 2023-0171). All the animal experiments were performed in accordance with ARRIVE 2.0 guidelines and regulations.

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III. RESULTS

1. Changes of average slow phase velocity of spontaneous nystagmus

Spontaneous nystagmus was developed immediately after labyrinthectomy. Slow phase velocity of spontaneous nystagmus significantly decreased over time from 1 to 12 hours after labyrinthectomy depending on the genotype of mouse and treated reagents (**Fig. 1**). In *Kcnq4*$^{+/+}$ mice, the average slow phase velocity was significantly slower in retigabine-treated mice than that in DMSO-treated mice at 7 days after labyrinthectomy; however, the average slow phase velocity was not different until 7 days after labyrinthectomy in *Kcnq4*$^{p.W277S/p.W277S}$ mice (**Fig. 1**).

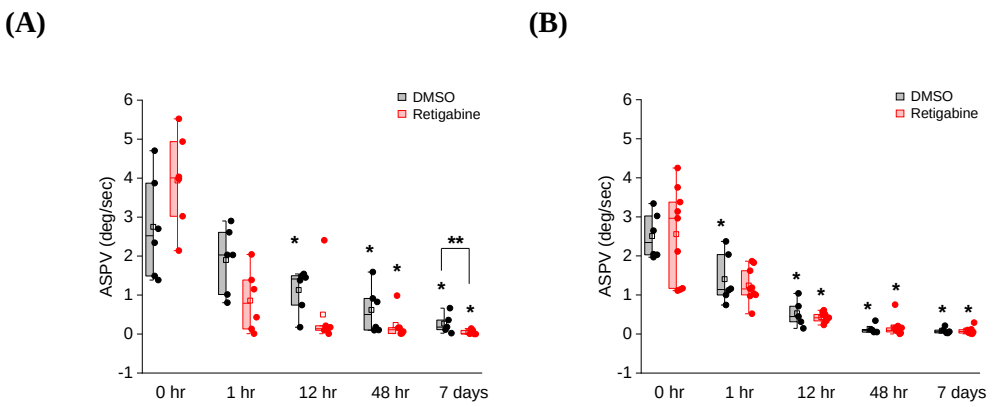

Figure 1. Changes of average slow phase velocity of spontaneous nystagmus after labyrinthectomy in *Kcnq4*$^{+/+}$ (A) and *Kcnq4*$^{p.W277S/p.W277S}$ mice (B).

AVSP, average slow phase velocity. DMSO, DMSO-treated mice; Retigabine, retigabine treated mice *, $p < 0.05$ comparison with 0 hr; **, $p < 0.05$, between DMSO and Retigabine.

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2. Changes of gain value in SHA test

Before performing labyrinthectomy, baseline gain in SHA test was measured. The average gain values at all the test freuqencies of mice treated with retigabine and DMSO were not significantly different. After labyrinthectomy, the gain values dropped significantly throughout all the test frequencies compared to the baseline in both groups **(Fig. 2)**. The gain value changes at each stimulation immediate after the surgery were not different between retigabine- and DMSO-treated group ($p>0.05$). The gain value at each stimulation frequency after the surgery was not different between the two groups until 48 hours, but the gain value at 1.28Hz was significantly higher in retigabine-treated group than that in DMSO-treated group ($p<0.05$).

In *Kcnq4*$^{p.W277S/p.W277S}$ mice, gain values were also significantly decreased after labyrinthectomy both in retigabine- and DMSO-treated group, however, there was no significant difference of the gain value at each stimulation frequency until 7 days after the surgery (**Fig. 3**).

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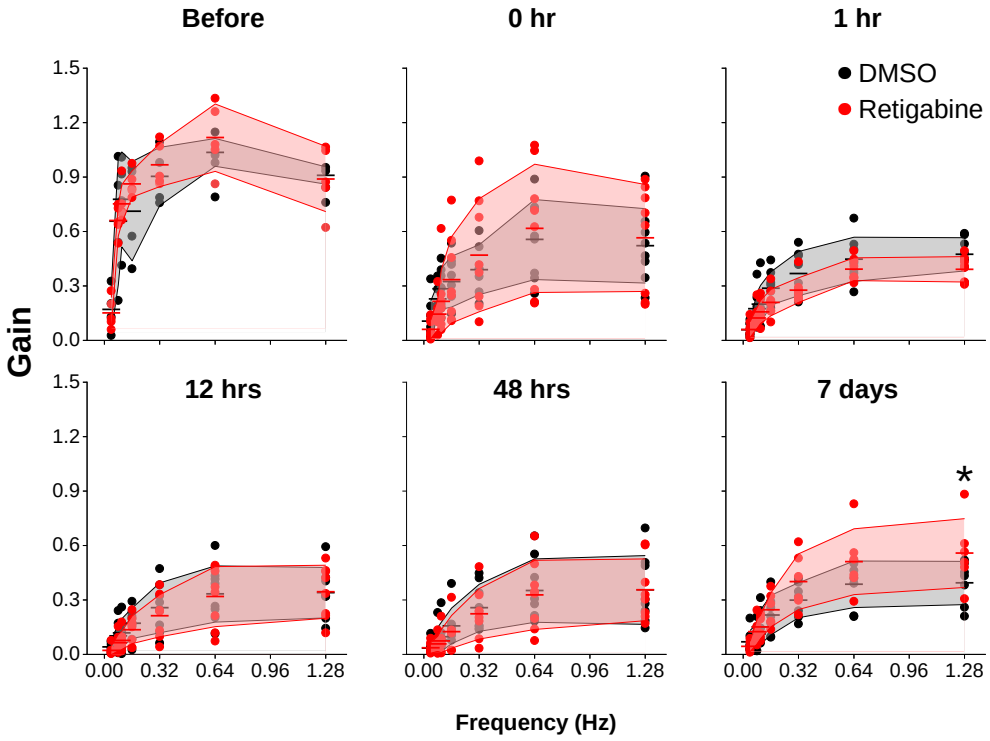

Figure 2. Time course of gain value change at each stimulation frequency after labyrinthectomy in *Kcnq4*$^{+/+}$ mice.

Before, before labyrinthectomy; 0hr, immediate after labyrinthectomy; DMSO, gain value in DMSO-treated mice; Retigabine, retigabine-treated mice; *, $p < 0.05$, between DMSO and Retigabine

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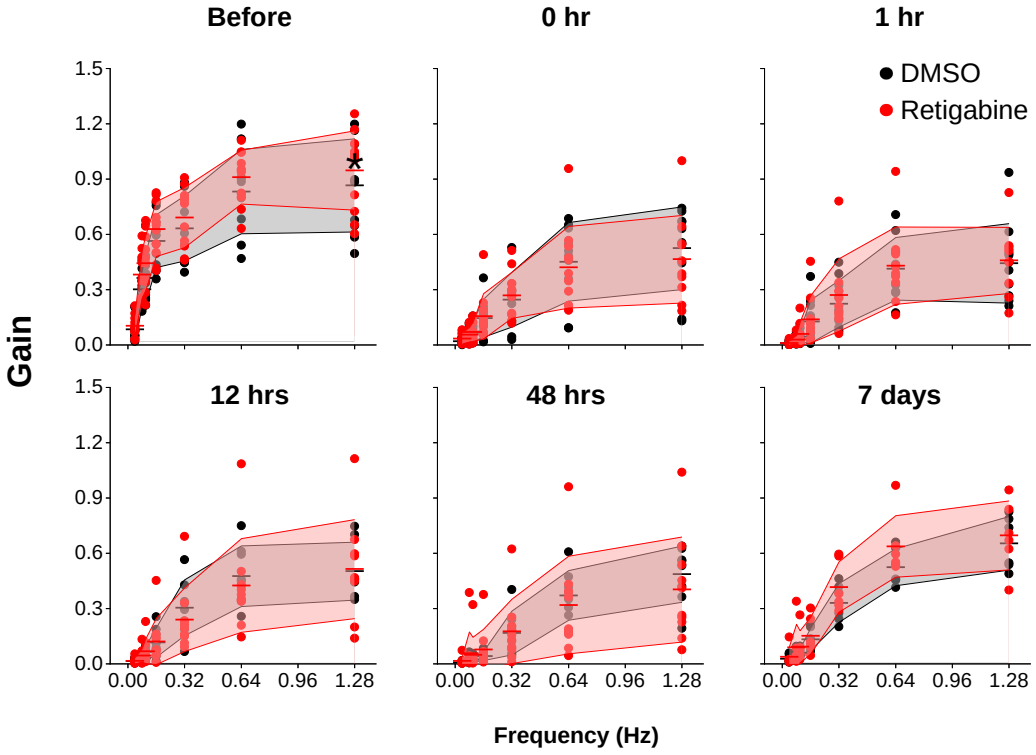

Figure 3. Time course of gain value change at each stimulation frequency after labyrinthectomy in *Kcnq4*$^{p.W277S/p.W277S}$ mice.

Before, before labyrinthectomy; 0hr, immediate after labyrinthectomy; DMSO, gain value in DMSO-treated mice; Retigabine, retigabine-treated mice; *, $p < 0.05$, between DMSO and Retigabine.

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3. Hair cell and KCNQ4-positive calyx changes after labyrinthectomy in vestibular periphery

We observed peripheral vestibular organs by confocal microscopy in *Kcnq4*$^{+/+}$ mice. In the left side, hair cell and KCNQ4-positive calyx were normal **(Fig. 5)**. In contrast, the right side, where labyrinthectomy was performed, showed severe loss of hair cells and calyx nerve endings **(Fig. 5)**. There was no difference in the loss of vestibular hair cells and calyx nerve endings between DMSO- and retigabine-treated mice **(Fig. 5)**.

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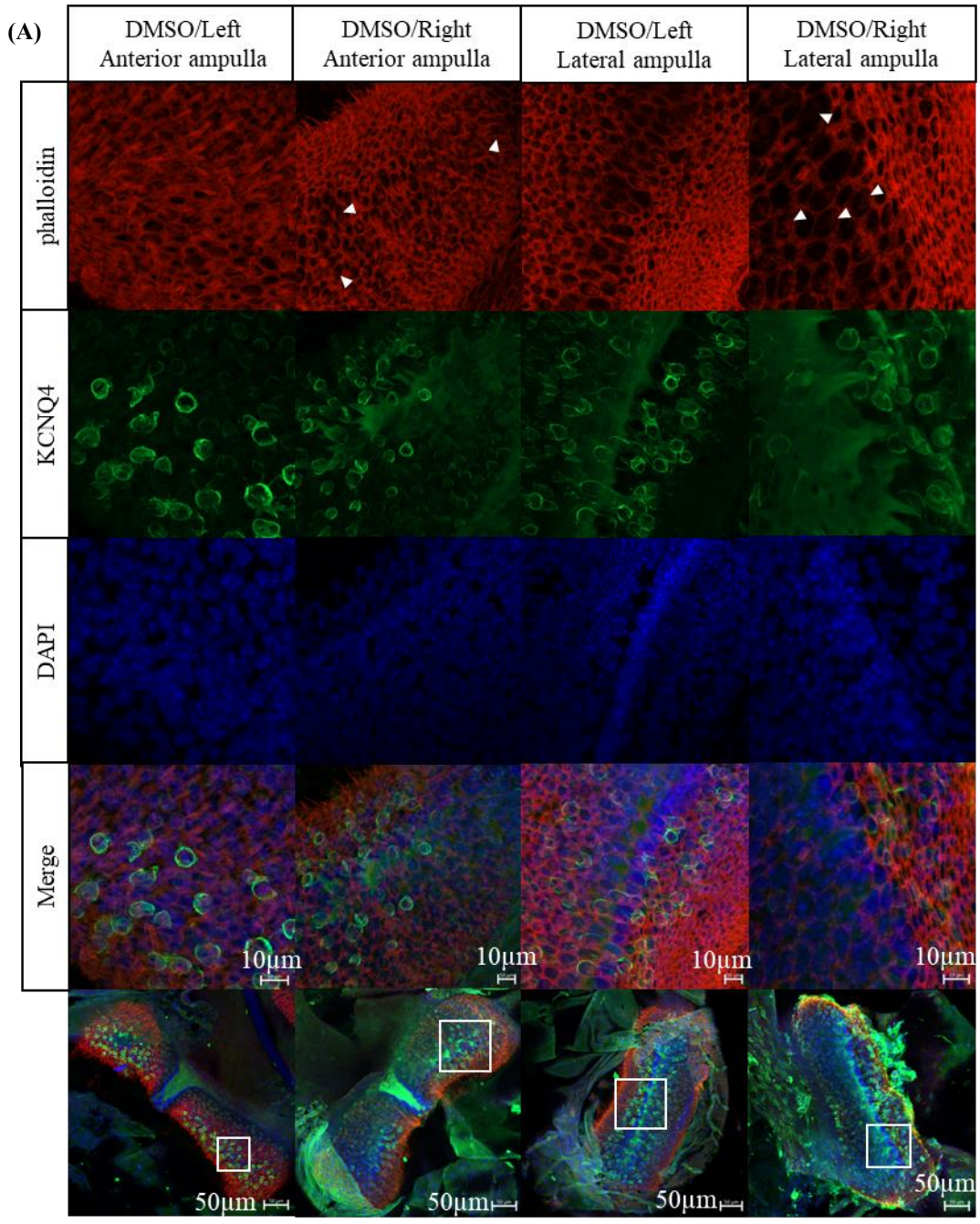

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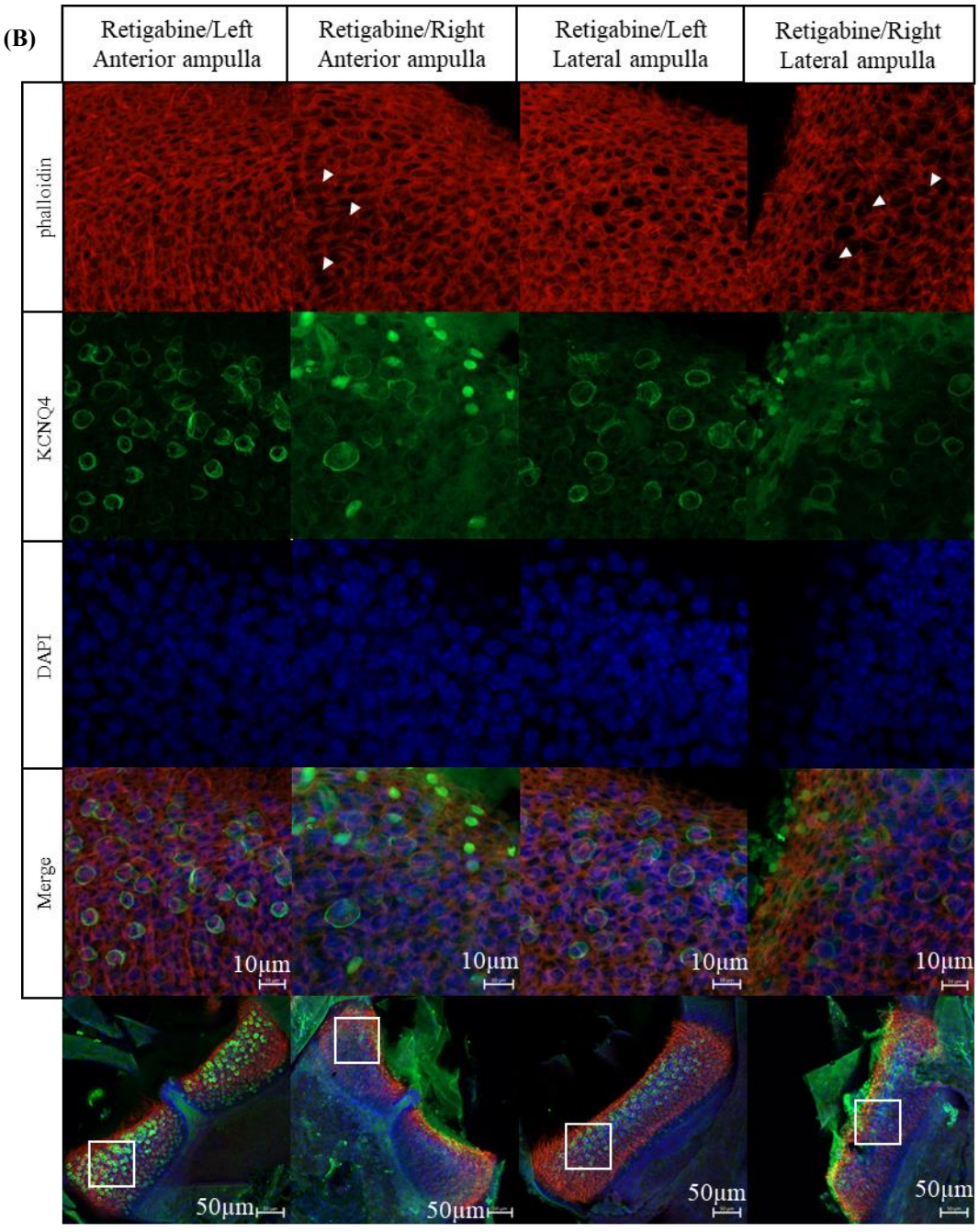

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Figure 4. Immunohistochemistry of vestibular organ after labyrinthectomy.

Vestibular endorgan comparison between DMSO (A) and Retigabine (B) groups. Both group's right side vestibular periphery showed severe loss of hair cells and KCNQ4-positive calyx nerve ending (triangle) when compared to the left side.

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4. KCNQ4 and c-fos expression in vestibular nucleus after labyrinthectomy

KCNQ4 was found to be located at DBA/2J Atlas - Section 32[7], where medial, superior, and lateral vestibular nucleus were located. In retigabine-treated mice, stronger expression of KCNQ4 in neuron cell bodies at medial vestibular nucleus (MVE) was found than that at MVE in DMSO-treated mice (**Fig 6**). c-fos was weakly expressed at MVE in DMSO-treated mice, however, the expression was stronger and prominent in retigabine-treated mice.

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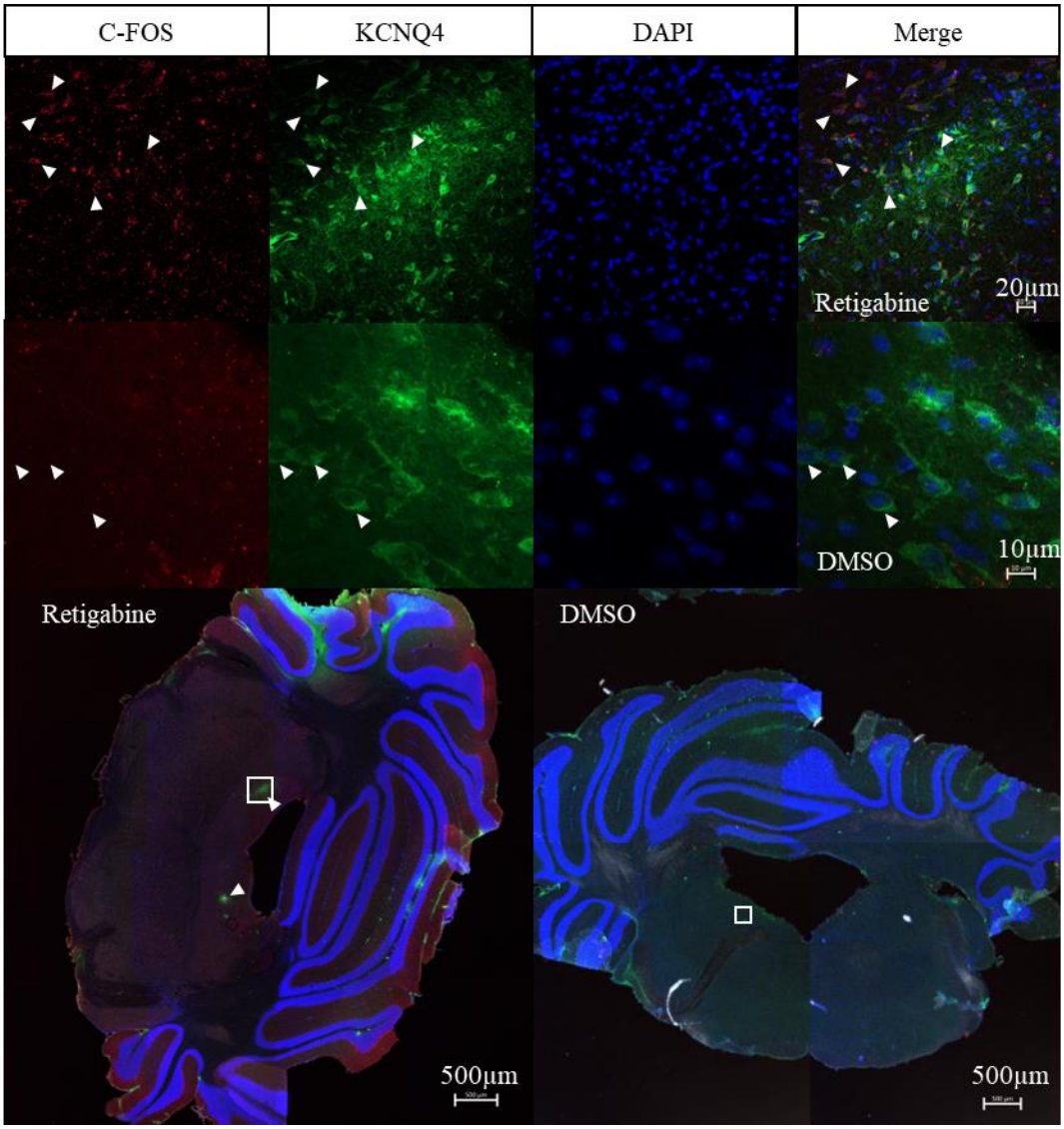

Figure 5. Immunohistochemistry of medial vestibular nucleus in brain stem.
c-fos and KCNQ4 expression was found at medial vestibular nucleus and their expression was stronger in retigabine-treated mice when compared to that in DMSO-treated mice.

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IV. DISCUSSION

In this study, we revealed the effect of KCNQ activator after acute unilateral vestibular loss. Most salient findings of this study can be summarized as follows. First, retigabine induced faster vestibular compensation, which was demonstrated by the decrease in average slow phase velocity of spontaneous nystagmus and increased gain value at 1.28Hz in SHA test. Second, KCNQ4 was found in the vestibular nucleus at brainstem level. Third, KCNQ4 activation in the vestibular nucleus was likely to be involved in the enhancement of vestibular compensation because KCNQ4 and c-fos expression was stronger in retigabine-treated mice.

After acute vestibular injury, imbalance of vestibular signals from bilateral vestibular endorgan is caused and unidirectional horizontal/torsional spontaneous nystagmus to healthy side develops due to the loss of vestibulo-ocular reflex in the lesion side. In rotation test, gain of vestibulo-ocular reflex decreases.[8] The coping strategy for the acute vestibular loss, vestibular compensation occurs immediately and progressively at the central vestibular system to maintain stable vision and body imbalance.[9] The mechanism of vestibular compensation was not revealed completely, but it was reported that vestibular tone at the vestibular nucleus was modified and adjusted through the connection of intercommisural fibers between vestibular nucleus.[10] As a result, intensity of spontaneous nystagmus decreases and finally disappears, and gain is recovered at mid to high frequencies. Vestibular rehabilitation which enhances vestibular compensation is a key approach in treating acute unilateral vestibular loss.[11] Although vestibular rehabilitation induces faster vestibular compensation when compared to the natural compensation, it usually takes several weeks to months depending on the degrees of vestibular loss, combined systemic disease conditions, and age. Therefore, it would be beneficial to the patients to return to daily life if there is a medication for faster enhancement of vestibular compensation.

Retigabine is an activator of KCNQ channels, and there were prior studies to improve hearing in patients with hearing loss and its effect is mainly on the activation of KCNQ4

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channels at the basolateral membrane of outer hair cells.[12] Because retigabine could also activate other KCNQ channels rather than KCNQ4 in the vestibular system, we tried to investigate if its acting site is mainly KCNQ4 by using mouse model with p.W277S mutation of KCNQ4, which showed cochlear and vestibular phenotype due to KCNQ4 dysfunction. If retigabine showed effect through the other KCNQ channels, the gain value and average slow phase velocity of spontaneous nystagmus should be improved *Kcnq4* $^{p.W277S/p.W277S}$ mice as well as *Kcnq4*$^{+/+}$ mice. The effect was only significant in *Kcnq4*$^{+/+}$ mice, therefore, KCNQ4 might be involved in the enhancement of vestibular compensation. This was also supported by the findings in immunohistochemistry of vestibular nucleus. c-fos protein expression has been used as a marker of neuronal activation because it monitors intracellular second messenger levels more precisely than simply neuronal activation. It was also used for studies of vestibular compensation after acute vestibular loss. FOS protein, the c-fos protein product, is a transcription factor that may represent a mechanism by which short-term signals at the cell membrane are transduced into long-term biochemical changes involving the synthesis of new proteins and leading to adaptive and/or compensatory process.[13] The expression of KCNQ4 and c-fos was stronger in the mice treated with retigabine. The finding suggested that retigabine induced KCNQ4 expression in the vestibular nucleus, which might be involved in the enhancing the vestibular compensation demonstrated by increased expression of c-fos. The contribution of KCNQ4 in the calyx nerve ending of vestibular endorgan was not likely to or minimally contribute to the enhancement of vestibular compensation because KCNQ4 in the calyx nerve ending was revealed to be destructed.

Although the results of this study provided evidence for the effect of KCNQ4 activator and involvement of KCNQ4 in vestibular compensation, there are several limitations. First, even though retigabine-treated mice showed improved gain at 7 days after labyrinthectomy, it was only significant at 1.28Hz stimulation frequency. The effect seemed to be limited and further evaluation of VOR gain more than 7 days will be needed in the future study. Second, we did not investigate the functional changes of KCNQ4 in the vestibular nucleus.

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If KCNQ4 was closely related to increased c-fos expression and enhanced vestibular compensation, KCNQ4 activity in the vestibular nucleus was increased. Blind patch clamp technique and current measurement combined with pharmacological method at vestibular nucleus will provide further direct evidence in the future. Also, there should be much more complex molecular mechanism related to KCNQ4 induced vestibular compensation process.[14] Molecular biologic studies should be combined with the functional studies and these kind of studies will provide basis for understanding the vestibular compensation mechanism and the development of new drug target for various vestibular disorders.

Even though our study has abovementioned limitations, we believe that the findings of this study will be a basic clue for understanding the role of KCNQ4 in the vestibular nucleus and for developing new therapeutic target, especially selective KCNQ4 activator, for acute vestibular loss.

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V. CONCLUSION

In unilateral labyrinthectomized mouse, a model of acute unilateral vestibulopathy, retigabine induced faster vestibular compensation acting on the KCNQ4 at vestibular nucleus. The findings of this study will provide a basis for understanding the role of KCNQ4 in the vestibular compensation and possibility for the development of target drug for acute vestibular loss.

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ABSTRACT(IN KOREAN)

급성 전정신경 기능 저하에서 KCNQ4 activator의 효과 규명

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내용
급성전정기능 저하는 현기증, 자발 안진 및 자세의 불안정을 유발한다. 현제까지는 뇌간의 전정보상으로 달성되는 시각적, 자세적 안정성 대신 전정기능회복을 위한 마땅한 치료법은 존재하지 않는다. KCNQ4는 전정유모세포의 구심성 신경에 분포하는 K+ 전압계폐 이온 채널이다. 전정계에서 KCNQ4의 역할은 아직 완전히 이해되지 않았다. 우리는 마우스 모델에서 급성 전정 손상에 대한 KCNQ4의 역할을 알아보고자 하였다. 우리는 일측성 미로 절제술로 전정기관이 손상된 마우스 모델을 이용하여, 수술 직후와 12시간 후 KCNQ4활성제인 retigabine과, 그 용매인 DMSO를 복강 내 주사하였다. 각 마우스 그룹에서 일측성 미로 절제술을 받은 후 전정기능의 변화는 정현파회전검사 및 전정안반사의 자발안진의 평균속도 검사를 이용하여 평가된다. 수술 직전, 각 약물을 주사한 후 1시간, 12시간(두번째 주사), 48시간 및 7일 후 시간에 따른 약물에 의한 전정보상 과정을 기록하였다. 이러한 과정을 $Kcnq4^{p.W277S/p.W277S}$ 마우스에서도 수행하며, KCNQ4 채널에 의한 retigabine의 특이적 효과를 확인하였다. $Kcnq4^{+/+}$ 마우스 그룹에선 48시간까지 정현파회전검사의 이득 값에 유의한 차이가 없었다. 그러나 retigabine을 주사한 그룹은 주사 후 7일째 1.28Hz의 고주파에서 유의하게 증가한 이득값을 보였다. 이와는 대조적으로 $Kcnq4^{p.W277S/p.W277S}$를 사용한 동일한 실험에서는 전정안반사에서 통계적으로 유의한 차이가 없었다. 정현파회전검사의 증가된 이득 값은 중추전정기관에서 KCNQ4의 활성화에 의해 유도된 중추전정보상작용의 증가일 가능성이 높을 것으로 예상된다. 중추전정기관의 역할을 확립하기

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위해, 우리는 c-fos를 사용했다. 뇌간에서 DNA 결합 인자인 c-fos의 발현 수준을 비교하여 DMSO와 retigabine 그룹의 차이를 발견할 수 있었다. 주사 후 3시간째, 우리는 마우스의 전정 기관을 고정하고 마우스의 뇌는 연수부위에 위치한 내측 전정 핵 주위를 동결절편했다. 림프액이 일측성 미로절제술에 의해 제거되었기 때문에, 오른쪽 전정 기관의 유모세포는 죽고 KCNQ4 채널은 약하게 발현되었다. 따라서 우리는 retigabine이 전정 기관에서 작용을 할 수 없고, 뇌간에서 작용한다는 결론을 내렸다. 우리는 내측 전정 핵에서 KCNQ4의 발현을 발견했고, retigabine 그룹은 DMSO 그룹보다 c-fos의 발현이 더 많았다. 결론적으로 retigabine은 내측 전정 핵의 KCNQ4 채널에 작용하여 일측성미로절제술로 인한 전정기능 장애 마우스 모델에서의 전정 보상수준을 증가시켰다.

핵심 되는 말 : 전정기능장애, 일측성미로절제술, Retigabine, KCNQ4, Kv7.4, 전정-안구 반사, 전정기능보상, 뇌간, 내측 전정 핵, C-FOS