이학석사 학위논문

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서울대학교 대학원

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이 논문을 이학석사 학위논문으로 제출함

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Study of PKMζ expression mechanism and the analgesic effect by PKMζ inhibition in neuropathic pain

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Seoul National University

Biological Sciences Major

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Abstract

Study of PKMζ expression mechanism and the analgesic effect by PKMζ inhibition in neuropathic pain

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Protein kinase M ζ (PKMζ) is a key molecule for maintaining neuropathic pain. Previous study has shown that neuropathic pain induces expression of PKMζ in anterior cingulate cortex (ACC) and inhibition of PKMζ by zeta inhibitory peptide (ZIP) reduces

behavioral sensitization in neuropathic pain model mice. However, the mechanism how PKMζ is expressed by peripheral nerve injury and whether treatment of ZIP is also effective in chronical pain model is not well studied. Here, we showed that PKMζ is expressed without relying on mRNA transcription in peripheral nerve injured mice. We also found that inhibition of PKMζ by ZIP is also effective for reducing behavioral sensitization in neuropathic pain lasting for one month. Our study is expected to be the basis for the development of treatment for chronic pain.

Keyword : Neuropathic pain, Chronic pain, Anterior Cingulate Cortex (ACC), Protein kinase M ζ (PKMζ), analgesic effect

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Introduction

The various functions of the brain are regulated by synaptic plasticity (Citri and Malenka, 2008; Ho et al., 2011). Especially in the process of memory formation, synaptic plasticity is known as the physical substrate of memory (Kandel, 2001; Choi et al., 2018). This synaptic plasticity has a variable property (Abraham and Bear, 1996; Citri and Malenka, 2008). The question of how to keep a memory for a long time although the synaptic connection is not permanent had been under investigation.

Protein kinase M ζ (PKMζ), the non-canonical isoform of protein kinase C, has recently emerged as a strong candidate (Lisman, 2017; Sacktor, 2011; Ko et al., 2016). PKMζ is a one of protein kinase enzyme that regulates other protein by phosphorylation (Hernandez et al., 2003). Unlike other forms, PKMζ does not have a regulatory domain and therefore has constitutively active feature (Serrano et al., 2005). Previous studies showed that PKMζ is

sufficient and necessary for Long-term potentiation, which is the cellular mechanism of memory (Ling et al., 2002). Application of PKMζ into postsynaptic neuron induces synaptic potentiation (Ling et al., 2002). Moreover, PKMζ inhibition by zeta inhibitory peptide (ZIP) impairs LTP in the hippocampus and several types of memories (Serrano et al., 2005; Pastalkova et al., 2006).

Neuropathic pain by peripheral nerve damage is also regulated by the synaptic plasticity (Buonomano and Merzenich, 1998; Jain et al., 1998; Merzenich et al., 1984; Zhuo, 2008). For example, tail amputation or nerve ligation induces LTP in the anterior cingulate cortex (ACC) (Li et al., 2010; Kang et al., 2012; Kuner and Flor, 2016; Ko et al., 2018). Since the molecular mechanisms of LTP are almost universal, previous investigators hypothesized that LTP induced by neuropathic pain would also be maintained by PKMζ and revealed that peripheral nerve ligation increases the expression of PKMζ in the ACC (Li et al., 2010). Moreover, they also found that inhibition of the PKMζ in the ACC by ZIP treatment successfully induced the analgesic effect (Li et al. 2010). However, whether the increase of PKMζ expression is transcription-dependent has not been studied. In addition, it remains unclear how long the analgesic

effect by the ZIP treatment lasts and whether PKMζ inhibition also induces analgesic effects in long-lasting chronic pain.

In this study, we investigated PKMζ mRNA and protein level in the ACC after LTP induction or peripheral nerve damage. We also examined the effect of ZIP treatment on the chronically maintained pain and checked the time of analgesic effect by ZIP treatment in the ACC.

Materials and methods

Animals

6~12 weeks old male C57BL/6NCrljBgi mice were used in all experiments (from Orient Bio). All mice were maintained under a 12-hr light/dark cycle and provided food and water by ad libitum. All experiments were conducted according as the guideline and regulations from the Institutional Animal Care and Use Committee (IACUC) of Seoul National University.

Cannula implantation surgery and drug infusion

Mice were anaesthetized by a ketamine/xylazine mixture and positioned in a stereotaxic apparatus. The ACC (AP: +0.7 mm, ML: ±0.4 mm, and DV: −1.7 mm) was targeted and the 24 gauge guide cannula were implanted bilaterally. After cannula implantation, the mice had a rest period at least one week. Injection cannula (30 gauge) was used for infusion into the ACC. ZIP (0.5μl, 10nmol/μl)

or actinomycin D (ActD) (20 ng/μl), or vehicle was infused bilaterally into the ACC within 1 minute and then the injection cannula stayed in the target region for 1 minute.

Nerve ligation surgery

The mice were anaesthetized by a ketamine/xylazine mixture. Artificial tears were used to protect their eyes. The left leg was shaven by the scissors and sterilized by povidone iodine liquid and alcohol. The left thigh skin was cut to expose the muscles. The muscles were incised using scissors and treated with sterile saline. Then common peroneal nerve (CPN) was ligated by a wax coated 4-0 silk suture. After the ligation, the incised skin was sutured by 5-0 silk suture.

Mechanical allodynia response

The mice were placed in separate chambers and habituated for 1 hour before experiments. Mechanical allodynia response was measured based on the reactivity of the hind paw against applying the von Frey filament to the point of bending. Mechanical allodynia was tested nine times with 5 minute intervals using 1.65 filaments.

Sudden withdrawal, biting and liking were considered as the positive responses. The mice then rested for 2 hours after drug infusion and the test was restarted.

PSD fraction

Nerve ligated mice were decapitated 2 hours after drug infusion. Three slices of the ACC near the drug infusion site were collected and six slices were used for PSD fraction. Frac buffer (4mM EDTA, 1 mM EGTA and 30 mM pH 7.4 Tris-Cl) with the protease inhibitor cocktail was used for homogenization. Then, the debris and nucleus fraction were removed by centrifugation (500g for 5 minute, twice) and supernatants were used for centrifugation at 100,00g for 1 hour. The pellet was lysed with Frac buffer including the protease inhibitor cocktail and 0.5% Triton X-100. The lysates were incubated for 20 minute in the ice and placed onto the 1M sucrose. After centrifugation at 100,000g for 1 hour, the pellet was lysed with PSD lysis buffr (5 M NaCl, 10% sodium deoxycholate, 1 M pH 7.4 HEPES, 10% sodium dodecyl sulfate (SDS), 100 mM DTT and 10% Triton X-100) including the protease inhibitor cocktail.

Western blot

Three slices of the ACC region were homogenized with RIPA buffer (150 mM NaCl, 50 mM pH 7.6 Tris-Cl, 0.1% SDS, 1 mM EDTA, 1% NP-40, 1 mM DTT and 0.5% sodium deoxycholate) including protein phosphatase inhibitor cocktail and protease inhibitor cocktail after LTP induction. Electrophoresis was conducted on 4-12% SDS-polyacrylamide gel (Invitrogen) and the proteins were transferred to a nitrocellulose membrane. The membranes were blocked by 3% bovine serum albumin or 5% skim milk in Tris buffered saline with Triton X-100 for 2 hours and incubated with primary antibody (PKCζ: 1:500, Invitrogen, phosphor-PKCζ: 1:1000, Cell Signalling, actin: 1:5000, Sigma) for one day. Next, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody. Immunoblots were analyzed by ChemiDoc™ MP System (Bio-Rad).

Quantitative real-time PCR

Three slices of the ACC (400 μm thickness) were dissected and then RNA was purified using RNAiso plus (TAKARA) or Trizol (Invitrogen). DNase I was treated for 15 minute and then purified

RNA was used to synthesize the cDNA. To examine the difference of the PKMζ mRNA levels, quantitative real-time PCR was conducted with SYBR premix Ex Taq II (TAKARA) using a CFX96 Real-Time PCT Detection system. Samples were analyzed by quantitative real-time PCR using the following primers: PKMζ (Forward: 5′ -ACGCCCACCTTCGGTAGAGC-3′ , Reverse: 5′ -GGACGTGGCAGCGTTTATGG-3′), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Forward: 5′ -TGCACCACCAACTGCTTA-3, Reverse: 5′ -GGATGCAGGGATGATGTTC-3)

LTP recording with *MED64*

The brain was placed in oxygenated cutting solution (124mM NaCl, 3mM KCl, 26mM NaHCO3, 1.25mM NaH2PO4, 10mM MgSO4, 15mM Glucose, and 2mM CaCl2) Coronal slices of the ACC were prepared by a vibratome (Leica VT 1000S) and placed in oxygenated artificial cerebrospinal fluid (ACSF) (124mM NaCl, 3mM KCl, 26mM NaHCO3, 1.25mM NaH2PO4, 1mM MgSO4, 15mM Glucose, and 2mM CaCl2) for at least 2 hours. After recovery, extracellular field excitatory postsynaptic potential (fEPSP) slopes in the ACC were recorded using Multielectrode array system

(MED64). Recording was performed according as previous report (Kang et al., 2012). 0.02Hz frequency stimulation was delivered and baseline recording was performed for 30 minute. After the baseline recording, 1mM Glycine (Tocris Bioscience) was applied for 30 minute and washed out. The data was collected from 4-6 channels near the stimulation sites for stable response.

Statistical analysis

Experiments data were analyzed using one-way ANOVAs or unpaired or paired t-tests. All data are shown as the mean±SEM. In all cases, statistical significance was considered by $*p < 0.05$, $**p < 0.01$.

Results

LTP stimulation in the ACC enhances PKMζ expression, but not transcription.

Previous studies showed that LTP stimulation increases PKMζ and p- PKMζ levels in the hippocampus and neuropathic pain induces a condition similar to LTP in the ACC (Kelly et al., 2007; Li et al., 2010). Therefore, we investigated whether LTP induction in the ACC also enhances PKMζ and p-PKMζ levels. We induced chemical LTP in the ACC using 1mM glycine treatment. Accordance with the previous study, we successfully induced chemical LTP after the glycine treatment (Figure 1). We found that PKMζ level showed a tendency to increase and p-PKMζ was significantly increased by the LTP stimulation (Figure 2). However, unlike the amount of protein, the levels of PKMζ mRNA were significantly reduced by the LTP stimulation (Figure 3). These results imply that neuropathic pain induces LTP-like state in the ACC and then

increase PKMζ expression without transcription.

Neuropathic pain enhances PKMζ expression without de novo new mRNA transcription.

Next, we investigated whether PKMζ expression by neuropathic pain needs mRNA transcription. We examined the PKMζ mRNA level in the ACC after nerve ligation surgery and found that the mRNA level of PKMζ did not change significantly (Figure 4, left panel). We also checked mRNA level using quantitative real-time PCR and there is no difference between control and nerve injury group (Figure 4, right panel). In addition, we revealed nerve ligation also enhances PKMζ expression even after transcription inhibitor ActD treatment (Figure 5). These results indicate that the increase of PKMζ induced by neuropathic pain is independent of mRNA transcription.

Analgesic effect by ZIP treatment appears only in limited time.

Allodynia response in neuropathic pain was reduced by PKMζ inhibition with ZIP (Li et al., 2010). Therefore, it is important to know how long the ZIP treatment effect will last in order to develop drugs to reduce neuropathic pain. We investigated the allodynia response at 4 or 6 hours after ZIP treatment into the ACC. We found that there was an analgesic effect 4 hours after ZIP treatment but no analgesic effect after 6 hours (Figure 6).

Analgesic effect by ZIP treatment also appears in chronically maintained pain.

The previous study tested the analgesic effect of the ZIP 3 or 7 days after nerve ligation surgery (Li et al., 2010). Since neuropathic pain is one of the types of chronic pain, it is important to make sure that ZIP is also effective in long-lasting neuropathic pain. Thus, we examined the effect of ZIP in mice that received nerve injury a month ago and showed that ZIP was still effective to reduce allodynia response in chronically maintained pain (Figure 7).

Figures

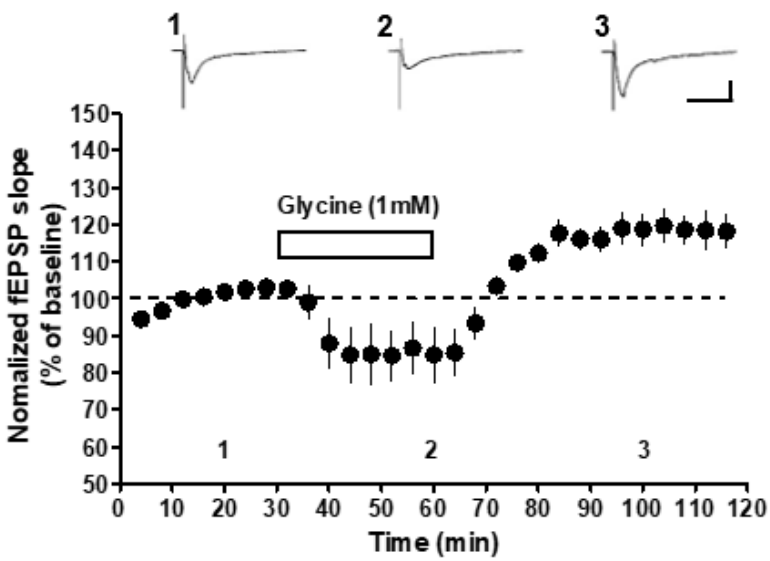

1
2
3
Glycine (1mM)
Nomalized fEPSP slope
(% of baseline)
150
140
130
120
110
100
90
80
70
60
50
1
2
3
0
10
20
30
40
50
60
70
80
90
100
110
120
Time (min)

Figure 1. Chemical LTP induced by the glycine treatment in ACC.

Application of 1mM Glycine successfully induced chemical LTP after wash out (n = 6 slices / 5 mice)

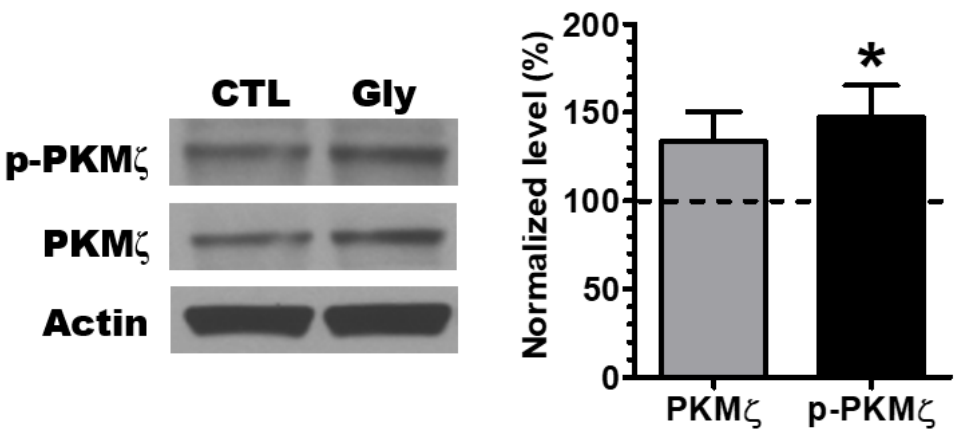

CTL
Gly
p-PKMζ
PKMζ
Actin
Normalized level (%)
200
150
100
50
0
*
PKMζ
p-PKMζ

Figure 2. The levels of PKMζ and p-PKMζ were increased by chemical LTP

The representative image of western blot (left panel). Western blot for PKMζ and p-PKMζ using the ACC slices induced chemical LTP by glycine treatment (right panel, PKMζ; control: 100.0 ± 2.6%, glycine: 133.6 ± 16.9%, n = 6-8, p = 0.09, p-PKMζ; control: 100.0 ± 6.9, glycine: 147.3 ± 17.9%, n = 5-6, unpaired t-test; *p < 0.05).

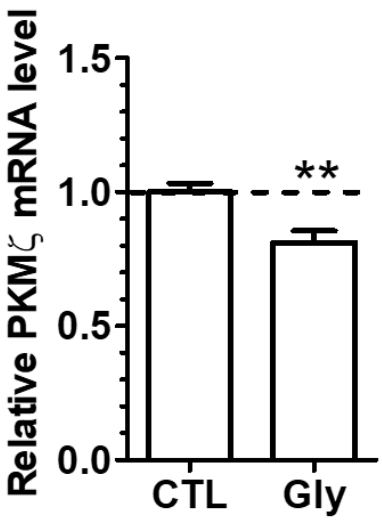
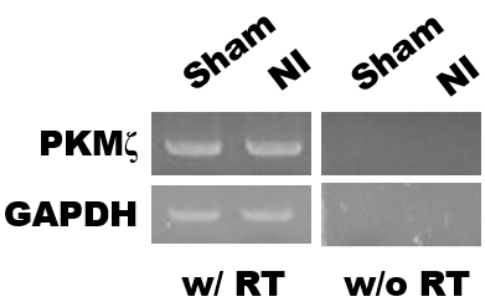

Relative PKMζ mRNA level
1.5
1.0
0.5
0.0
**
CTL
Gly

Figure 3. The PKMζ mRNA levels were reduced by chemical LTP in ACC slices.

(A) The mRNA levels of PKMζ were significantly reduced by glycine treatment (CTL: control, 1.000 ± 0.08167%, Gly: Gylcine, 0.8115 ± 0.04356%, n = 6, unpaired t-test; **p < 0.01).


Sham
NI
Sham
NI
PKMζ
GAPDH
w/ RT
w/o RT

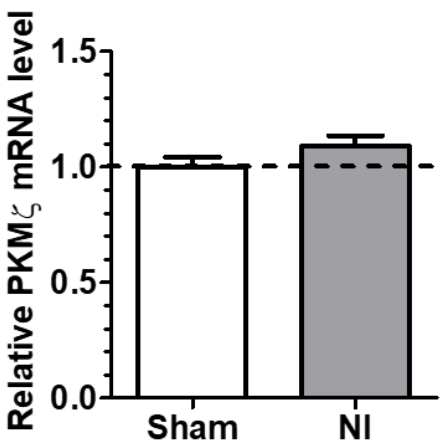
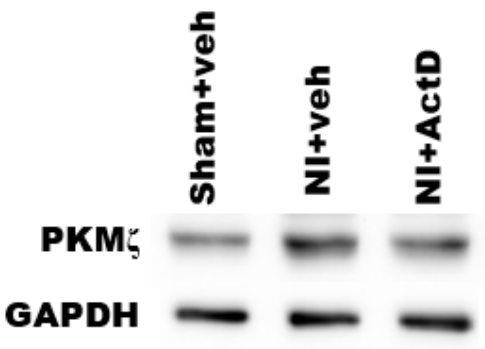

Relative PKMζ mRNA level
1.5
1.0
0.5
0.0
Sham
NI

Figure 4. The PKMζ mRNA levels were not changed in the ACC by nerve injury.

The PKMζ mRNA levels in the ACC 3 days after nerve injury. GAPDH was the internal control (left panel, NI: nerve injury). The mRNA levels measured by quantitative real-time PCR were not changed after nerve injury (right panel; n = 10-11 per group, unpaired t-test; $p > 0.05$).


Sham+veh
NI+veh
NI+ActD
PKMζ
GAPDH

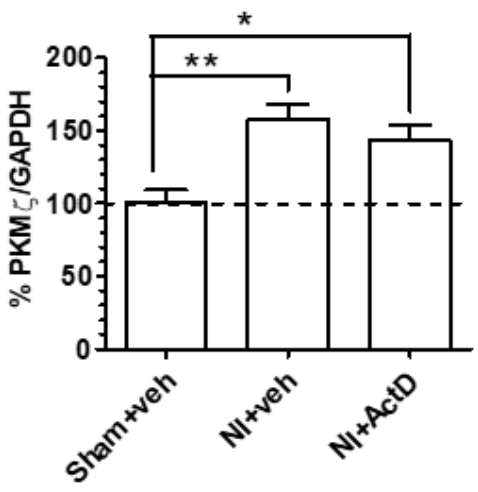
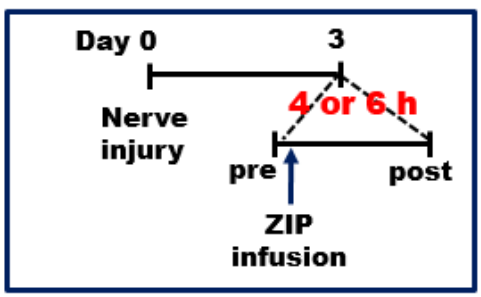

*
**
200
150
100
50
0
% PKMζ/GAPDH
Sham+veh
NI+veh
NI+ActD

Figure 5. Peripheral nerve injury enhances PKMζ expression and transcription inhibition dose not reverse PKMζ expression.

The representative image of western blot (left panel). Nerve injury enhanced the PKMζ expression. Transcription inhibitor ActD did not prevent the enhancement of the PKMζ expression (Sham + veh: 100 ± 9.403%, NI + veh: 157.3 ± 10.50%, NI + ActD: 143.3 ± 10.33%, n = 8–9 mice per group, $p < 0.01$, one–way ANOVA followed by Tukey's multiple comparisons post hoc test. *$p < 0.05$; **$p < 0.01$).


Day 0
3
Nerve injury
4 or 6 h
pre
post
ZIP infusion

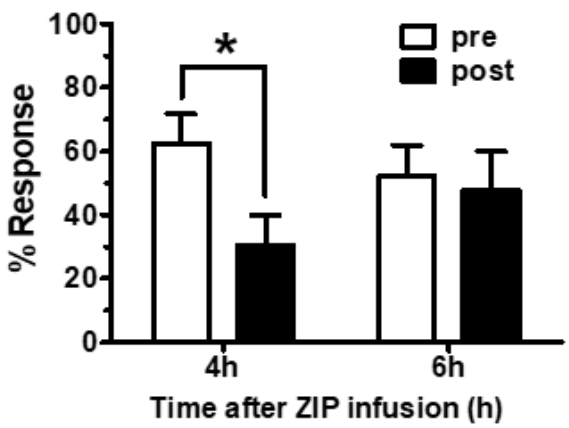
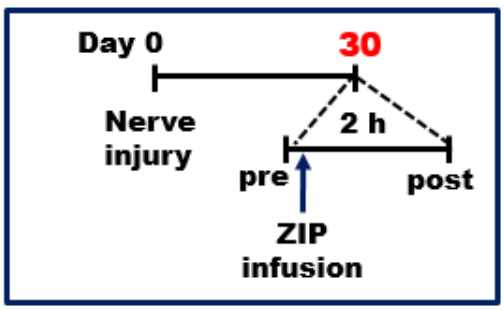

100
80
60
40
20
0
% Response
*
pre
post
4h
6h
Time after ZIP infusion (h)

Figure 6. The analgesic effect by ZIP treatment appears after 4 hours, but not after 6 hours.

Schematic showing the experimental protocol (upper panel). The mice were tested for mechanical allodynia response three days after peripheral nerve injury. Before ZIP infusion, the allodynia response was examined (Pre). Then, the allodynia response was examined again at 4 or 6 hours after ZIP treatment ($n = 4–5$, paired t−test; $*p < 0.05$).


Day 0
30
Nerve injury
2 h
pre
post
ZIP infusion

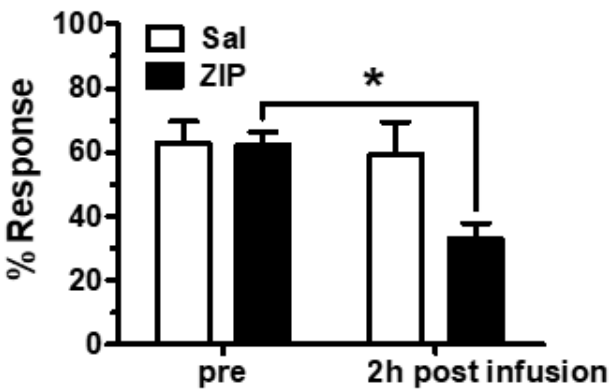

100
80
60
40
20
0
% Response
Sal
ZIP
*
pre
2h post infusion

Figure 7. The analgesic effect by ZIP treatment also appears in the pain lasting for one month.

Schematic showing the experimental protocol (upper panel). The mice were tested for mechanical allodynia response a month after peripheral nerve injury. Before ZIP infusion, the allodynia response was examined (Pre). Then, the allodynia response was examined again at 2 hours after ZIP treatment (n = 3–5 per group, unpaired t–test between saline and ZIP infusion group, paired t–test between "pre" and "2 h post infusion" in the ZIP infusion group; *p < 0.05).

Discussion

In this study, we found that LTP stimulation or neuropathic pain increases PKMζ and p-PKMζ levels in the ACC and these increases do not depend on the mRNA transcription (Figure 2, 3, 4, 5). Moreover, we showed that ZIP treatment is also effective for analgesic effect in chronically maintained pain, but only for a certain period of time (Figure 6, 7). Although PKMζ mRNA level was not changed 3 days after nerve damage, it does not mean PKMζ expression does not require the transcription at all. It might be possible that transcription level is rapidly increased in a short time after the nerve injury. After then, PKMζ and p-PKMζ levels are enhanced and maintained for a long time for an unknown reason. Therefore, in order to examine the exact mechanism of the expression, it is necessary to study the amount of PKMζ mRNA and protein in several time point.

In order to use ZIP as a medicine, it is necessary to show the efficiency of the ZIP treatment. We found that ZIP treatment is

effective for reducing mechanical allodynia response in chronically maintained neuropathic pain. Considering the previous study showing that ZIP infusion into the ACC did not disturb other brain functions such as anxiety or memory, PKMζ could be the therapeutic target (Li et al. 2010). However, we also showed that ZIP treatment is only effective in a limited time. So in order to make PKMζ a therapeutic target, it is necessary to develop a system capable of continuously delivering ZIP or an inhibitor that can inhibit PKMζ for a long time.

Pain is caused by not only peripheral nerve injury but also other stimuli such as tissue damage or disease (Merskey et al., 1986; Cleeland and Ryan., 1994). Our study investigated the role of PKMζ only in neuropathic pain. It might be possible that other pain responses are also regulated by PKMζ. To use the ZIP as the medicine for a painkiller, it is necessary to show that PKMζ is also upregulated by the other pain stimuli and ZIP is also effective in reducing other types of pain. In addition, previous studies have shown that the ACC is activated by itch situation and ACC is associated with the itch related behavior (Hsieh et al., 1994; Darsow et al., 2000; Mochizuki et al., 2003; Ikoma et al., 2006;

Leknes et al., 2007). As itch and pain have similar features, PKMζ might be regulated in the ACC under the processing of the itch response (Stander and Schmelz., 2006; He et al., 2016). Therefore, further studies should be done for the role of PKMζ in other pain responses or itch situation.

In this study, we showed that the PKMζ expression is independent of the transcription in nerve injury mice and investigated the efficiency of the PKMζ inhibition using the ZIP. Our research will contribute to the study of drugs that alleviate neuropathic pain.

This study is published in the journal of '*Molecular pain*' and data were re-used (Ko et al., 2018).

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Ko, H. G., Ye, S., Han, D. H., Park, P., Lim, C. S., Lee, K., Zhuo, M., and Kaang, B. K. (2018). Transcription-independent expression of PKM ζ in the anterior cingulate cortex contributes to chronically maintained neuropathic pain. Molecular pain, 14, 1744806918783943.

국 문 초 록

PKMζ 단백질은 신경병성통증을 유지하고 조절하는데 중요한 분자이다. 이전 연구에서 신경병성통증은 전대상피질에서 PKMζ의 발현을 유도하고, 또한 제타 억제 성 펩티드 (ZIP)를 통한 PKMζ의 억제는 신경병성통증 모델 마우스에서 진통효과를 낸다는 것을 보여주었다. 그러나 기존의 연구에서는 말초 신경 손상에 의해 PKMζ가 어떻게 증가되는지, 그리고 ZIP을 통한 치료가 오랫동안 지속된 통증 모델에서도 효과가 있는지에 대해서는 연구가 이루어지지 않았다.

본 연구에서는 말초 신경 손상 모델 마우스의 전대상피질에서 PKMζ가 mRNA 전사에 의존하지 않고 PKMζ의 발현이 증가되는 것을

확인하였다. 또한 ZIP에 의한 PKMζ의 억제가 1개월 동안 지속 된 신경병성통증에서도 효과가 나타난다는 것을 확인 하였다. 또한 ZIP 처리에 의한 진통 효과는 4시간 까지는 지속 되지만, 6시간 뒤에는 나타나지 않는 것을 보여주었다. 이러한 연구는 추후 진행 될 만성통증 치료제 개발 연구에 도움을 줄 것이라고 기대 된다.

주요어 : 신경병성통증, 만성 통증, 전대상피질, PKMζ, 진통 효과 ,

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