이학석사 학위논문

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김 시 용 의 이학석사 학위논문을 인준함

2016년 12월

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Studies on chemogenetic inhibition of the ACC excitatory neurons in a mouse model of inflammatory hyperalgesia induced by CFA

Advisor: Professor Bong-Kiun Kaang, Ph.D.

A dissertation submitted to the Graduate Faculty of Seoul National University in partial fulfillment of the requirement for the Degree of Master of Science

Kim Siyong

Interdisciplinary Program in Neuroscience

Graduate Scholl of Natural Sciences

Seoul National University

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Abstract

Studies on chemogenetic inhibition of the ACC excitatory neurons in a mouse model of inflammatory hyperalgesia induced by CFA

Siyong Kim

Interdisciplinary Program in Brain Sciences

The Graduate School

Seoul National University

Many previous studies have shown that the anterior cingulate cortex (ACC) is involved in cognitive/emotional pain processing. Nonetheless, because of the heterogeneous composition and projections in the ACC, the underlying central mechanism that modulates chronic pain is still unclear. However, recent rodent model studies have demonstrated that the excitatory/inhibitory and temporal optogenetic modulation of excitatory neurons in the ACC can induce/alleviate hyperalgesia. Together with optogenetics, the designer receptors exclusively activated by designer drugs (DREADDs) system, which is

chemogenetic tool, has been utilized for the long-term activation/inhibition of neuronal activities in many brain regions. Here, we first show that the chemogenetic inhibition of excitatory neurons in the ACC can reduce mechanical pain responses. To examine this, we injected inhibitory DREADD (hM_4D_i)-expressing adeno-associated virus (AAV) into the mouse ACC. After recovery from the surgery, we subcutaneously injected complete Freund's Adjuvant (CFA) into the right hind paw of mice to induce hyperalgesia and measured the changes in mechanical thresholds with electronic von Frey apparatus. Additionally, we calculated the ratio of c-Fos-positive hM_4D_i-mCherry- or mCherry-expressing neurons to total hM_4D_i-mCherry or mCherry-expressing neurons. By analyzing the changes in mechanical thresholds and relative neural activities represented by c-Fos, we demonstrated that reducing neural activity in excitatory neurons in the ACC could directly alleviate hyperalgesia induced by CFA. To our knowledge, this is the first study that has utilized chemogenetics to study chronic pain in the supraspinal region. In addition, these findings extend the available research tools for examining chronic pain. Moreover, this study successfully reproduced the results of studies on optogenetics.

Keywords: Chronic pain, hyperalgesia, CFA, DREADDs, hM_4D_i, von Frey test, anterior cingulate cortex

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Introduction

Anterior cingulate cortex and its role in nociception

Because of its clinical relevance and importance, pain has been a major topic of discussion for several decades based on both clues from animal model studies and from functional imaging studies using fMRI in humans. According to the International Association for the Study of Pain, chronic pain is defined as a state of pain that persists past the healing phase following an injury, and it is usually caused by inflammation (inflammatory pain) and injuries in the nerves (neuropathic pain). Additionally, it is associated with mood disorders, such as anxiety and depression (Blackburn-Munro and Blackburn-Munro, 2001). Accompanying these two forms of chronic pain, hyperalgesia is a sensitized behavioral response to noxious stimuli (Zhuo, 2014).

As well as the peripheral processing of pain, the central processing of pain, including modulations in the dorsal horn and supraspinal regions, has been extensively debated. Although the specific neural circuit involved in chronic pain is not clearly understood yet, cumulative evidence points toward the forebrain structure, the anterior cingulate cortex (ACC), as an important site for endogenous pain control in terms of pain modulation through cognitive intervention, such as attention and anticipation (Bantick et al., 2002; Petrovic et al., 2002; Ploghaus et al., 1999; Porro et al., 2003). Furthermore, several studies have reported

that increased activation of the ACC in both acute and chronic pain situations has been observed, suggesting that the ACC plays a crucial role in responding to noxious stimuli (Bliss et al., 2016; Shyu et al., 2008; Sikes and Vogt, 1992; Vogt, 2005; Zhuo, 2008).

The ACC's role in affective pain has also been consistently studied. A report demonstrates that ACC activity is necessary and sufficient for noxious stimuli to produce an aversive memory (Johansen and Fields, 2004), suggesting it plays a role in pain-related perception (Apkarian et al., 2004; Zhuo, 2006).

Despite these previous studies, the exact role of the ACC in pain is still controversial because several groups have reported that the activation of the ACC is associated with rather reduced pain behavior (Fuchs et al., 2014; LaBuda and Fuchs, 2005).

Long-term potentiation and chronic pain

The characteristics of the development and maintenance of chronic pain are similar to those of long-term potentiation (LTP), which is the representative model of neural plasticity. Thus, the genetic deletion and pharmacological inhibition of LTP-related molecules have been suggested as key models for explaining chronic pain (Apkarian et al., 2009; Li et al., 2010; Wang et al., 2011).

LTP-dependent hypersensitivity was reported in a study on the deletion of GluA1, a postsynaptic glutamate receptor subunit, in the dorsal root ganglion (Gangadharan et al., 2011). Many studies of the ACC exhibit similar results. Both pre- and postsynaptic modifications have been reported in inflammatory pain and neuropathic pain (Bie et al., 2011; Qiu et al., 2013; Wu et al., 2005). Furthermore, Zhao et al. (2005) reported the importance of postsynaptic Ca^{2+} signaling through the chelating method in the mouse ACC (Zhao et al., 2005). In particular, in several studies of inflammatory pain, enhanced synaptic transmissions were observed with arthritis and complete Freund's Adjuvant (CFA) injection (Bie et al., 2011; Zhao et al., 2006).

However, recent studies have demonstrated that pre- and postsynaptic LTP play different roles in the pain process. Based on a behavioral study, presynaptic LTP is related to anxiety induced by chronic pain (Koga et al., 2015). In contrast, postsynaptic LTP seems to be a key cellular substrate for chronic pain, but not anxiety (Zhuo, 2016).

DREADDs system

Designer receptors exclusively activated by designer drugs (DREADDs) have recently been widely adopted as a powerful tool for the dissection of neural circuits (Garner et al.,

2012; Mahler et al., 2014; McCall et al., 2015; Vazey and Aston-Jones, 2014). They utilize the inertness of clozapine-*N*-oxide (CNO), a designer drug, for normal subjects (Armbruster et al., 2007). DREADDs have successfully shown the ability to control individual G protein-coupled receptors (GPCRs) signaling in many studies, from research on yeast to primates (Farrell and Roth, 2013; Urban and Roth, 2015).

Among DREADDs, hM_4D_i is an inhibitory $G_{i/o}$-coupled receptor that induces hyperpolarization and the silencing of transient neuronal activity by activating inwardly rectifying potassium 3 (Kir3) channels (Armbruster et al., 2007). This inhibitory DREADD is capable of being paired with phenotype-specific viral vectors; thus, it silences or attenuates neuronal activities without cell function distortion (Becnel et al., 2013; Ferguson et al., 2011; Guettier et al., 2009; Krashes et al., 2011).

CNO can take about 2 hours, to be cleared from plasma. It exerts its maximum effect on DREADDs 30 minutes after administration. This temporal window, however, enables DREADDs to modulate long-term neuronal modulation in a reversible manner (Guettier et al., 2009).

Recent studies on pain using optogenetics and chemogenetics

The optogenetic modulation of neuronal activity can control in several millisecond scales. As well as the ACC, the

prelimbic and infralimbic areas are also of interest in pain model studies (Lee et al., 2015; Wang et al., 2015; Zhang et al., 2015). Zhang et al. (2015) demonstrated that activation of prefrontal parvalbumin (PV)−positive neurons can induce affective pain. Wang et al. (2015) state that contralateral activation of prelimbic excitatory neurons decreases chronic pain and these neurons are deactivated after CFA injection. A lesion in Cg1 (the dorsal part of the ACC), however, does not alter the pain response. This is a somewhat conflicting conclusion to that of Kang et al. (2015). They showed that the selective activation of pyramidal neurons in the ACC can elicit an analgesic effect in an animal model of inflammatory pain.

Another group also used optogenetics to stimulate the inhibitory neural circuitry of the ACC. They found that activation of the circuit successfully attenuated pain behavior in a formalin test using Thy1−Channelrhodopsin2 (ChR2) mice (Gu et al., 2015). However, another group published a report arguing that optogenetic stimulation of the ACC sufficiently induces anxiety− and depressive−like behavior (Barthas et al., 2015). The discrepancy between these studies seems to be due to the indiscriminate expression of ChR2 in the ACC of Thy1−ChR2 mice.

Iyer et al. (2016) used chemogenetic modulation (DREADDs) with the combination of modified channelrhodopsin in a pain study. They demonstrated that the inhibition of spinal

neurons through activation of inhibitory DREADDs can successfully increase mechanical and thermal pain thresholds (Iyer et al., 2016).

To our knowledge, no study has utilized chemogenetics in the mouse ACC on inflammatory pain. Therefore, we tested whether the inhibition of excitatory neurons in the mouse ACC through hM_4D_i reduces hypersensitivity after developing inflammatory pain induced by a single injection of CFA in the peripheral region. We used an electronic von Frey test to show changes in the mechanical threshold and immunohistochemistry analysis to represent changes in neuronal activities.

Methods

Animals

For the entire study, adult (7–12 weeks old) αCaMKII–Cre hetero mice (Jackson Laboratory) and their wildtype littermates were used. All animals were housed under a 12 h light/dark cycle with food and water provided *ad libitum*. All works were conducted according to the policy for the care and use of laboratory animals approved by the Institutional Animal Care and Use Committee at Seoul National University

Stereotaxic virus injection

AAV carrying EF1a–DIO–hM$_4$D$_i$–mCherry or EF1a–DIO–mCherry construct was injected into 7–week–old αCaMKII–Cre hetero male and female mice. The intraperitoneal injection of ketamine–xylazine (0.1mg per gram of body weight of ketamine, 0.01 mg per gram of body weight of xylazine) was administrated for the anesthetization of each mouse, and the head was fixed in stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA).

Before injecting viral constructs, two holes were drilled in the bilateral ACC for the virus injection. The virus was delivered using a 10–μl syringe (Hamilton) and a 30–gauge metal needle. The injection volume and flow rate were 0.5

μl/site at 0.1 μl/min, and they were monitored by an injection pump (WPI). The virus was injected into both sides of the ACC (anteroposterior [AP] + 1.0 mm from bregma, mediolateral [ML] ± 0.35 mm, dorsoventral [DV] −2.2 mm). After injection, the needle was left for an additional 7 min and was then slowly removed. After surgery, the scalp was sutured and each animal was sent to their home cage for recovery for 4−5 weeks.

Whole−cell patch−clamp recordings

At the level of the ACC, coronal brain slices (300 μm) were obtained with a vibratome (Leica VT1200, Buffalo Grove, IL) in ice−cold oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 25 $NaHCO_3$, 1 NaH_2PO_4, and 10 glucose with oxygenation (9595% O_2, 5% CO_2). The brain slices were transferred to a submerged recovery chamber containing oxygenated ACSF for at least 1 hour at room temperature. The recording pipettes (3−5 MΩ) were filled with intracellular solution containing (in mM) 124 K−gluconate, 5 NaCl, 1 $MgCl_2$, 0.2 EGTA, 10 HEPES, 2 MgATP, 0.1 Na_3GTP, and 10 phosphocreatine disodium (adjusted to pH 7.2 with KOH). The experiments were performed in a recording chamber on the stage of a microscope.

For the recordings in the ACC, eEPSPs were recorded from layer II/III neurons with an Axon 200B amplifier. In the

current clamp experiment, after stabilization, a small current was delivered through a patch pipette to maintain a resting membrane potential at −65 mV for all recorded cells. Depolarizing step current (800 ms) with an intensity of 100 pA was injected once through the recording electrode, which induced consistent action potentials. CNO (100 μM) was administrated 30 min before the current−clamping experiment. A pyramidal cell with membrane potential less negative than −85 mV was excluded from the recording experiments (Cao et al., 2009).

Electronic von Frey test

Electronic von Frey apparatus (Ugo Basile, Italy) was placed underneath the testing grid to assess the mechanical threshold of hind paw. A transparent plastic chamber was laid on the grid.

On the first testing day (Day 0), the baseline of the withdrawal mechanical threshold was measured through the stimulation of hind paw. The threshold was measured six times with intervals of 6 min. CFA injection into the right hind paw of each mouse was conducted after determination of the basal level of response (Sigma−Aldrich F5881, 10 μl of 50% (v/v) CFA in saline).

On the third day after CFA injection (Day 3), the mice were acutely anesthetized by isoflurane, followed by the

intraperitoneal injection of Clozapine-*N*-oxide (CNO, Enzo Life Sciences BML-NS105, 10 mg/kg in saline) or saline after 2-3 hours of habituation in the testing chamber. For testing the inertness of the CNO itself, saline was administrated to wild-type littermates. The mice were then returned to the testing chamber with an additional 40 min of acclimation. For the following 80 min, the paw withdrawal threshold was once again with electronic von Frey apparatus. The mechanical threshold was measured six times in every mouse with 6min inter-trial intervals (ITIs). The highest value and the lowest value were excluded from the calculation, and the rest of the values were averaged.

Immunohistochemistry staining c-Fos

Within 2 hours of CNO injection, the mice that finished electronic von Frey test were acutely anesthetized with isoflurane and underwent transcardial perfusion with 10-mM phosphate-buffered saline (PBS: pH 7.4), followed by 4% paraformaldehyde (PFA) in PBS. The prepared whole brains were soaked in 4% PFA solution at 4 ° C overnight, and media was exchanged with 30 % sucrose the next day. The brains were dehydrated in 30% sucrose solution for 2 days at 4 ° C in a dark room. After fixative processes, the brains were sliced (40-μm thickness) in a frozen state with OCT compound in a cryostat at

under −20 ℃ (Leica CM3050S, Buffalo Grove, IL). From each animal, sections from the ACC (approximately 1.5 mm rostral to the bregma) were selected for the c−Fos immunohistochemistry and kept in 50% glycerol in PBS.

The obtained brain slices were washed in PBS three times for 5 mins, followed by a preblocking process with 5% normal goat serum for an hour. The sectioned slices were then incubated at 4 ° C overnight with a rabbit polyclonal antibody against c−Fos (1:200 dilution; sc−52; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a dark room to avoid the photobleachings of endogenous fluorescent protein (mCherry). The next day, the sections were rinsed three times with PBS, incubated for 2 hours in a secondary antibody, Alexa 488−conjugated goat anti−rabbit antibody (1:200 dilution; A−11034; Invitrogen), with covered state, After incubation, the slices were washed with PBS. DAPI solution (10 ug/ml, D1306, Thermo Scientific) was applied to sections for 5 mins followed by PBS washing. The sections were mounted on a glass slide, air−dried, and cover slipped, and then image acquisitions was performed using a confocal microscope (Leica Microsystems, Wetzlar, Germany).

Quantitative analysis of c−Fos expression

To determine whether neuronal activities in the ACC

influenced the mechanical paw withdrawal thresholds, we manually counted c-Fos-positive (c-Fos(+)) cells within the mCherry-positive (mCherry(+)) population in the blind state and calculated the relative ratio of c-Fos expressions (c-Fos(+)/mCherry(+)) using ImageJ after performing 5x magnification confocal microscopy.

Statistics

Statistical comparisons were performed using the unpaired *t*-test and two-way ANOVA by GraphPad Prism5 and SigmaPlot 10. Repeated measurements of two-way ANOVA with a Bonferroni post-test electronic von Frey test were performed to compare the effects of CNO administration in behavioral test. In all cases, statistical significance was represented by $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. All data are presented as mean ± SEM.

Results

Inertness of CNO to electronic von Frey test in naïve mice

Although the previous study (Armbruster et al., 2007) showed that CNO can only activate hM_4D_i without influencing the activities of other naïve GPCRs and though the mutation sites of inhibitory DREADD are evolutionary conserved (Armbruster et al., 2007), these experiments were demonstrated in *in vitro* experiments in yeast and cultured human neurons. Furthermore, CNO itself is a representative metabolite of clozapine in human (Gauch and Michaelis, 1971), and reports have found that CNO might be a weak antagonist of $5-HT_{ic}$ receptor in the choroid plexus of the rat (Kuoppamaki et al., 1993a; Kuoppamaki et al., 1993b). Therefore, the potential implications of CNO for inflammatory hypersensitivity were accessed first. The mechanical paw withdrawal threshold of two groups of αCaMKII-Cre wildtype littermates (CNO vs. saline) were measured by using electronic von Frey test **(Fig. 1)**. On Day 3, the mechanical thresholds after administration of CNO vs. saline were compared. There were no differences between CNO (n=11) and saline (n=10) in terms of mechanical thresholds in electronic von Frey test. Both the saline and CNO groups presented lowered mechanical sensitivity to a similar degree (*post hoc* Bonferroni t-test, $p > 0.05$) **(Fig. 2)**. These results prove CNO does not distort the nociception behavior in normal mice.

Attenuation of action potentials induced by CNO administration.

To test if activations of inhibitory DREADDs were capable of silencing or attenuating neuronal activities in the ACC, we conducted whole-cell patch recording in a slice from a mouse expressing hM_4D_i-mCherry (hM4Di) **(Fig. 3A)**. It revealed that pyramidal neurons in the ACC of the hM4Di mouse had a reliable resting membrane potential (approximately −65 mv) and generated a series of evoked action potentials (APs) (seven) during the step-current injection. After CNO administration, the resting membrane potential of the pyramidal neuron was hyperpolarized at about −7 mV accompanying attenuated generations of evoked APs (four) **(Fig. 3B)**. In summary, it was demonstrated that CNO successfully activated the hM_4D_i receptors, which resulted in attenuated neuronal activities as previously reported (Ferguson et al., 2011).

Electronic von Frey test in mice expressing hM_4D_i in ACC excitatory neurons

We tested whether the attenuating/silencing neuronal activities of excitatory neurons in the ACC restored mechanical hyperalgesia, which was induces by CFA. This was the working hypothesis of this study based on previous studies reporting increased excitatory transmission in the ACC after CFA injection (Qiu et al., 2013; Wu et al., 2005). We habituated each mouse in

the test chamber with transparent walls. After acclimation, the mice underwent intraperitoneal injection of CNO with acute anesthesia using isoflurane. Before awakening, the mice were returned to their test chamber and allowed 40 min of additional acclimation. Mechanical paw withdrawal thresholds were assessed six times with 6-min ITIs to prevent physiological adaptation or sensitization **(Fig. 1)**.

On Day 0, there was no difference in the baseline mechanical paw withdrawal threshold between mCherry-expressing mice (mCherry) (4.81 ± 0.09 g; n = 20) and hM4Di (4.94 ± 0.10 g; n = 19) (p = 0.507, *post hoc* Bonferroni t-test). CNO was administrated in both groups after inducing hyperalgesia by CFA on Day 3. This alleviated the mechanical threshold only in hM4Di mice (4.70 ± 0.16; n = 19), but not in mCherry mice(3.76 ± 0.19; n = 20) (p < 0.001, *post hoc* Bonferroni t-test). Two-way ANOVA revealed that the effect of inhibitory DREADD was significant (p = 0.002), and the interaction between the expression of hM_4D_i (DREADD) and the day after CFA injection (Time) was also significant (p = 0.0013) **(Fig. 4)**. This suggests that attenuating the neuronal activity of excitatory neurons in the ACC alleviates inflammatory nociception responses.

Immunohistochemistry for staining c-Fos in the ACC

We confirmed whether this increased mechanical

threshold is a representative behavioral phenotype resulting from reduced neuronal activities in the ACC. To track the neuronal activities, we employed immunohistochemistry assay to stain c-Fos. The neuronal activity and expression of c-Fos are closely correlated (Harris, 1998; Kawashima et al., 2014). This assay is also utilized in many pain studies not only on cortical neurons but also spinal neurons (Coggeshall, 2005; Narita et al., 2004). Furthermore, several studies have reported that administrating analgesic drugs suppresses nociception-induced c-Fos expression in the spinal cord (Gogas et al., 1991; Honore et al., 1995; Narita et al., 2004) and the ACC (Takeda et al., 2009).

Because the expression level of Fos protein peaks about 2 hours of gene transcription induction (Harris, 1998), we sacrificed mice from each group 2 hours later since the first trial for the measurement of the mechanical paw withdrawal threshold had been assessed. After behavioral measurement followed by fixative processes **(Fig 5A)**, immunohistochemistry staining targeting c-Fos was performed followed by confocal microscopy. The number of c-Fos(+) cells and mCherry(+) cells were manually counted in Cg1 and Cg2 based on anatomical criteria (Paxinos and Franklin, 2004) with the aid of ImageJ in the blind state **(Fig. 5B)**.

The relative neuronal activity was calculated as a ratio of c-Fos(+) cells and mCherry(+) cells (i.e., c-

Fos(+)/mCherry(+)). The relative c-Fos expression of hM_4D_i was statistically different from that of mCherry (p = 0.0002, unpaired t-test) **(Fig. 5C)**. Taken together with the behavioral results, this analysis revealed that the administration of CNO successfully inhibited the excitatory activities of neurons during the electronic von Frey test.

Figures

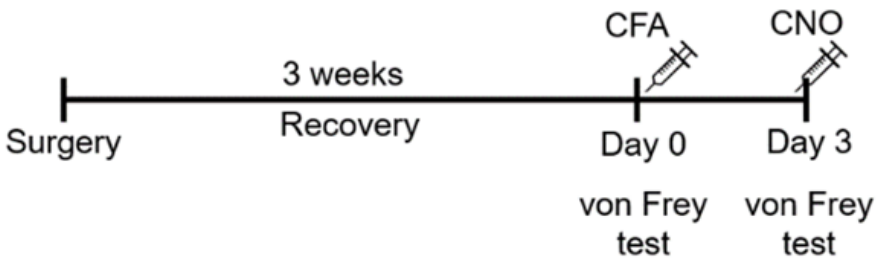
CFA
CNO
3 weeks
Recovery
Surgery
Day 0
Day 3
von Frey test
von Frey test

Figure 1. The overall experimental schedule. After recovery from virus injection surgery, each group of αCaMKII-Cre mice were tested for their basal level of mechanical paw withdrawal threshold, followed by subcutaneous CFA injection in the right hind paw on Day 0. On Day 3, CNO was delivered to each mouse and the mechanical threshold was measured for 80 min.

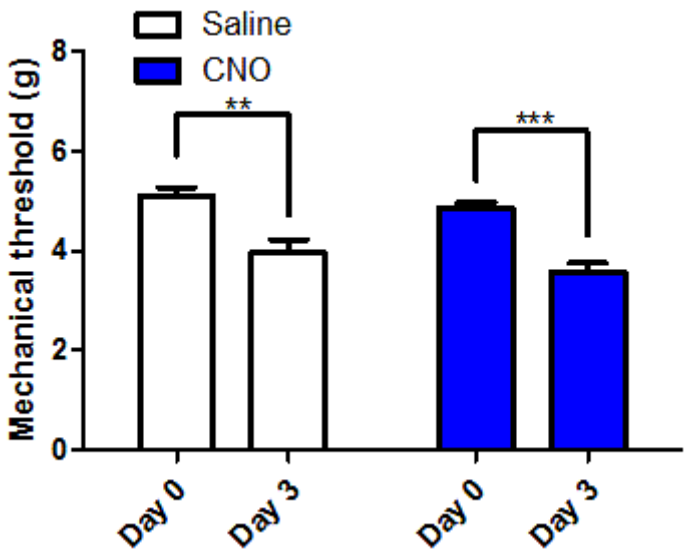
Saline
CNO
Mechanical threshold (g)
8
6
4
2
0
**
***
Day 0
Day 3
Day 0
Day 3

Figure 2. The inertness of CNO in the mechanical paw withdrawal threshold. On Day 0, the thresholds of the saline group (5.09 ± 0.18; n = 10) and CNO group (4.86 ± 0.11; n = 11) did not show significant differences (p = 0.2759, *post hoc* Bonferroni t−test). Likewise, On Day 3, both groups showed statistically insignificant differences in the threshold. ([saline]: 3.96 ±0.27; n = 10 vs. [CNO]: 3.57 ± 0.28; n=11, p = 0.2372, *post hoc* Bonferroni t−test, $^{**}p < 0.01$, $^{***}p < 0.001$). Two−way ANOVA revealed that only the effect of time was significant ($p < 0.0001$).

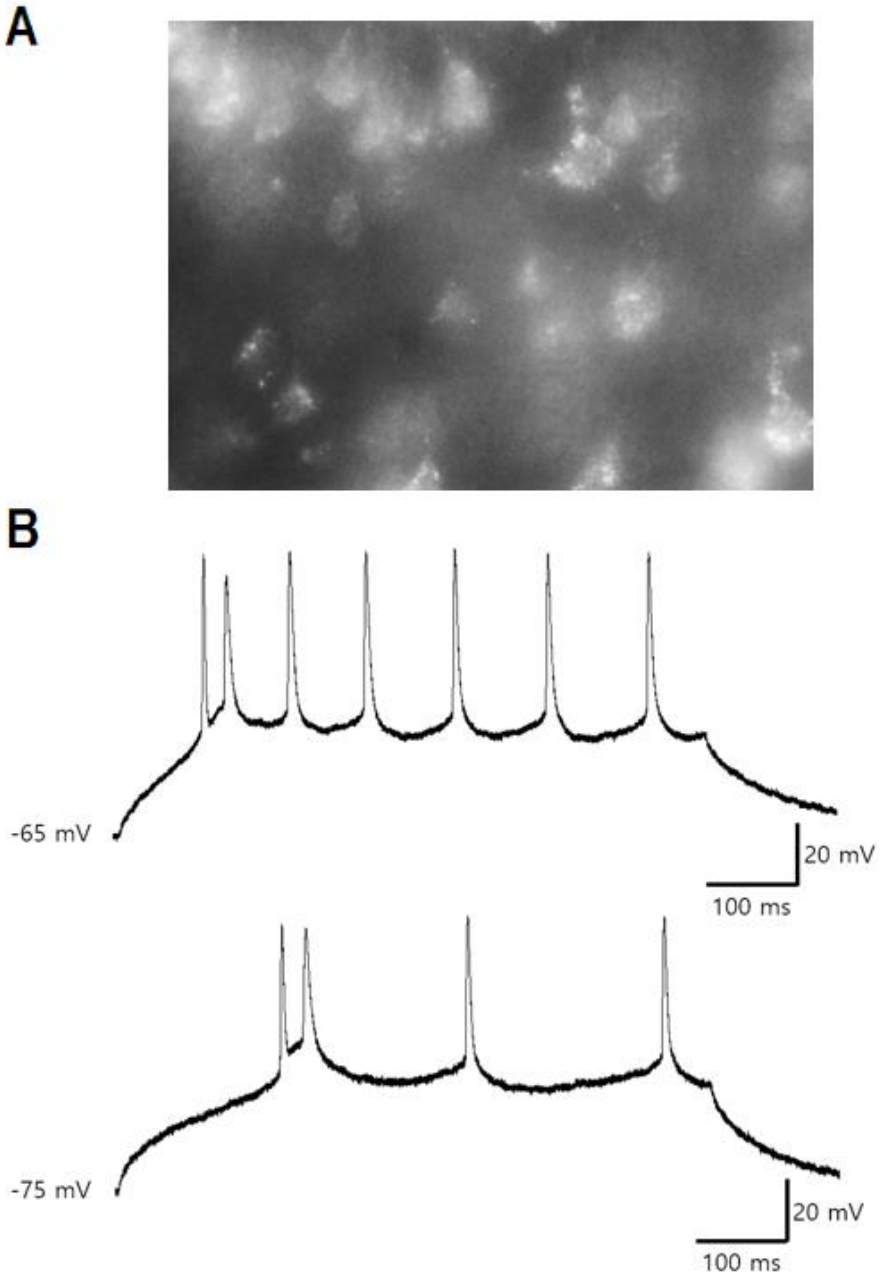
A
B
-65 mV
20 mV
100 ms
-75 mV
20 mV
100 ms

Figure 3. Functional validation of DREADD system. **(A)** Sample image of pyramidal cells expressing hM_4D_i–mCherry on the patch–clamp stage. **(B)** Representative recording of action potential firing of a pyramidal cell in response to current injection (100 pA) in brain slices from hM4Di mice. (Upper) Before CNO administration. (Lower) After CNO administration (100 μM, 30 min). CNO induced Kir3 mediated hyperpolarization, which resulted decreased the number of evoked action potentials in hM_4D_i–expressing neurons.

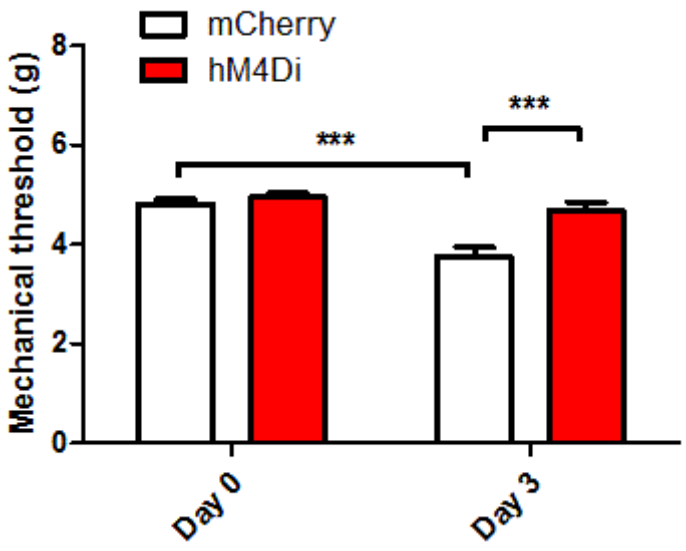
mCherry
hM4Di
Mechanical threshold (g)
8
6
4
2
0
***
***
Day 0
Day 3

Figure 4. Inhibitory DREADD effect on CFA-induced hyperalgesia. mCherry (4.81 ± 0.09 g; n = 20) and hM4Di (4.94 ± 0.10 g; n = 19) showed insignificant differences in threshold on Day 0 (p = 0.507, post hoc Bonferroni t-test). There was a significant difference in threshold on Day 3 between hM4Di (4.70 ± 0.16; n=19) and mCherry (3.76 ± 0.19; n = 20) (***p < 0.001, *post hoc* Bonferroni t-test). Two-way ANOVA revealed that the effect of hM_4D_i was significant (p = 0.002), and interaction between the expression of hM_4D_i (DREADD) and day after CFA injection (Time) was significant (p = 0.0013). Data are presented as mean ± standard error of the mean (SEM).

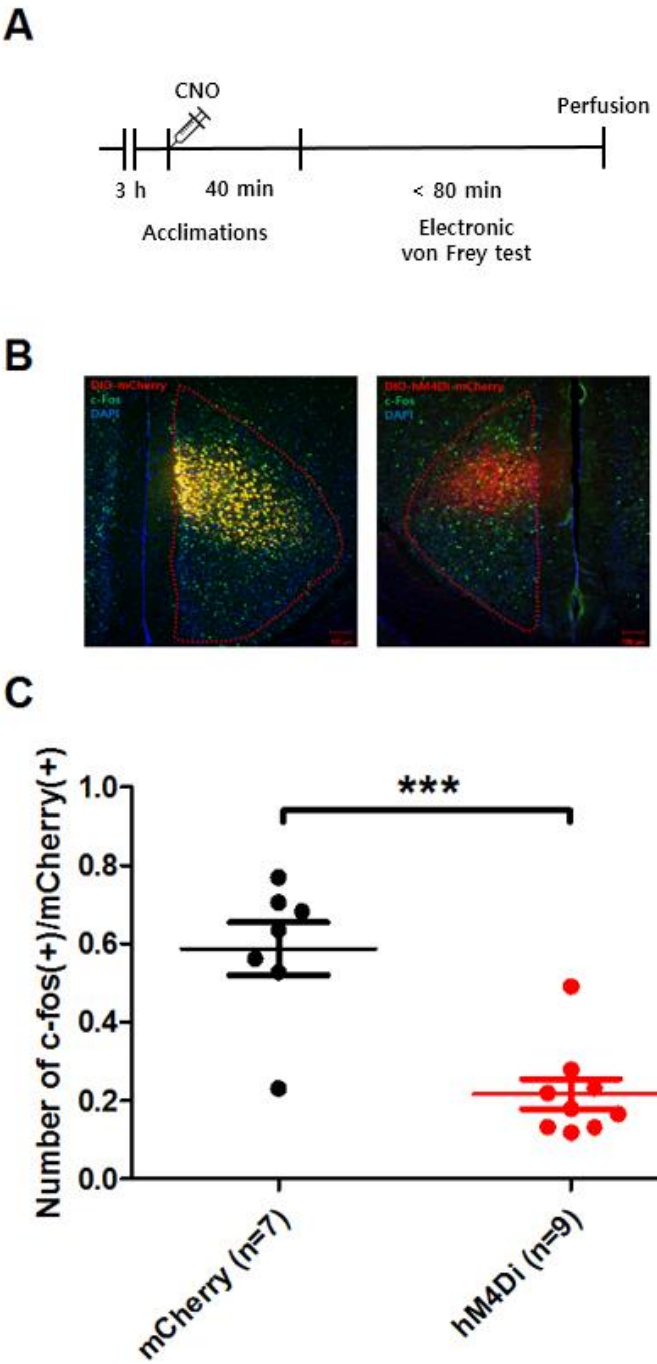
A
CNO
Perfusion
3 h
40 min
< 80 min
Acclimations
Electronic
von Frey test
B
C
Number of c-fos(+)/mCherry(+)
1.0
0.8
0.6
0.4
0.2
0.0
***
mCherry (n=7)
hM4Di (n=9)

Figure 5. Reduced c-Fos expression level through hM_4D_i activation in the ACC. **(A)** The schedule for behavioral assay and fixation **(B)** Representative confocal images of the ACC from (Left) mCherry mouse and (Right) hM4Di mouse, respectively. The red dashed line indicates boundaries of Cg1 and Cg2. Scale bar represent 100 μ m. **(C)** The relative c-Fos expression level was statistically different between mCherry (0.59 $\pm$ 0.07; n = 7) and hM4Di (0.22 $\pm$ 0.04; n = 9) (p = 0.0002, unpaired t-test). ***p < 0.001. Data are presented as mean $\pm$ standard error of the mean (SEM).

Discussion

In this study, we firstly reported the inertness of CNO on the mechanical paw threshold, and the analgesic effect of hM_4D_i-mediated attenuating neuronal activity of excitatory neurons in mouse the ACC. Hyperpolarized excitatory pyramidal cells generated a reduced number of action potentials. Additionally, this attenuation was reflected in the c-Fos expression level. Through Cre-mediated CaMKII-positive cell-specific inhibition of the ACC, we successfully reduced hypersensitivity induced by CFA. In terms of the analgesic effect, reduced c-Fos expression was observed, and these results are consistent with those of previous studies (Buritova et al., 1996; Gogas et al., 1991; Honore et al., 1995). In addition, we can conclude that attenuation by hM_4D_i activation successfully blocked postsynaptic activity, which is a crucial component of pain maintenance (Koga et al., 2015). However, it is unknown whether this attenuation can elicit long-term depotentiation.

Aside from the alleviation of CFA-induced hyperalgesia, there are limitations in this study. First, there are three types of excitatory neurons in the ACC. In neuropathic pain state, the population distribution of these neurons is changed (Cao et al., 2009). However, we transfected hM_4D_i to an intermingled population of excitatory neurons, which might have induced a complex effect in pain processing. Secondly, as well as pain

perception, pain memory and the affective aspects of pain are involved in the ACC (Barthas et al., 2015; Ikeda et al., 2013; Johansen and Fields, 2004; Li et al., 2009). Although we inhibited postsynaptic activity, which might be crucial for pain perception, we did not assessed any affective aspect of pain, or pain-induced anxiety. However, Kang et al. (2015) reported that the optogenetic excitation/inhibition of excitatory neurons in the ACC did not induce changes in anxiety-like behavior. Lastly, because DREADDs serve as a surrogate for endogenous GPCRs, they inevitably modulate and interfere with neuronal systems in an identical manner to that of endogenous GPCRs (Urban and Roth, 2015). Since G_i protein-coupled receptor is involved in the pain signaling in the spinal cord (Chen et al., 2009), cautious assessment is required for using DREADDs.

However, the DREADDs system have many advantages over optogenetics. Optogenetics can provide neuronal modulation on the millisecond timescale, and enable us to study neural circuitry through dissections. At the same time, however, it requires subsequent hardware attachment such as implanting a fiber optic cannula, which can be challenging as well as invasive. Optic fibers connected to the optic cannula can constrain the animal from moving freely, which can result in confoundable outcomes. Furthermore, inhibitory opsins are challenged with their potential side effects such as disruption of Cl^- gradients and pH changes (Mahn et al., 2016; Raimondo et al., 2012). On the

other hand, chemogenetic modulation, especially the DREADDs system used in this study, is useful for tonic modulation of specific neural circuits between a range of from a few minutes to hours (Vazey and Aston-Jones, 2013). Futthermore, the DREADDs can successfully mimic GPCR-activating signaling mechanisms that resemble neuronal modulation (Farrell and Roth, 2013). In this point of view, because brains with chronic pain undergo long-term modification of synapses while the animals are allowed to freely move, utilizing DREADDs for chronic pain study seems relevant.

Other brain regions such as the insular cortex and medial prefrontal cortex, are also promising targets for pain studies using DREADDs (Dunckley et al., 2005; Henderson et al., 2007; Lee et al., 2015; Zhang et al., 2015), since their roles in pain processing are not fully understood yet. In this study, we provided evidence that CNO does not alter nociceptive responses during inflammatory pain. At the same time, we introduced DREADDs into pain studies of the supraspinal region for the first time, thereby providing novel contributions to pain research. The utilization of DREADDs allows for tonic regulation of chronic pain circuits, and thus provides therapeutic contributions for clinical applications.

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초 록

마우스 화학유전학을 통한 전대상피질 흥분성 뉴런들의 억제가 CFA로 유도된 염증성 통각과민에 미치는 효과

김 시 용

기존의 많은 선행연구들은 전측 대상회 피질 (anterior cingulate cortex, ACC)가 통각의 처리과정에서 의식/감정적인 측면을 담당하고 있다고 보고해왔다. 하지만 많은 연구들에도 불구하고, ACC가 가지는 입력신호들의 다양함 때문에 만성통증을 조절하는 중추 메커니즘은 아직 불명확하게 밝혀진 상태이다. 최근 설치류 모델을 이용한 한 연구는 광유전학을 이용하여 ACC의 흥분성 뉴런의 활성/억제를 통해 통각의 유도/완화가 가능함을 보였다. 광유전학과 함께 널리 쓰이는 화학유전학 기술의 하나인 designer receptors exclusively activated by designer drugs (DREADDs)는 일시적인 조정을 가하는 광유전학 기술과 달리 한 번의 조작으로 상대적으로 더 긴 시간의 뉴런의 활동을 조정할 수 있는 기술이다. 이 연구에서는 최초로 ACC의 흥분성 뉴런들의 활성을 화학유전학인 억제가 기계적 통각자극에 대한 반응을 감소시킬 수 있음을 보였다. 이를 위해 억제성의 DREADDs의 한 종류인

hM_4D_i를 아데노 바이러스 (AAV)를 통해 쥐의 ACC 흥분성 뉴런들에 발현시켰으며, 회복 후, complete Freund's Adjuvant (CFA)를 주입하여 염증성 통각과민을 유도했다. 이후 여기서 일어나는 기계적 통각자극에 대한 역치의 변화를 von Frey test를 이용하여 추적했다. 또한 c-Fos 양성이면서 hM_4D_i-mCherry 양성이거나 mCherry 양성인 세포수의 비율을 계산했으며, 이 분석을 통해 기계적 통각 자극에 대한 역치의 증가가 c-Fos로 대변되는 신경세포들의 활성 감소로 인한 효과임을 보여주었으며, 따라서 ACC의 흥분성 뉴런들의 활성의 감소가 CFA로 인한 통각과민을 완화할 수 있음을 밝혔다. 이는 화학유전학을 이용하여 척수의 상위단계에서 만성통증을 연구한 첫 번째 사례이며, 이를 통해 만성통증을 연구하는 다양한 방법을 제공할 수 있었다. 동시에 이번 연구는 기존의 광유전학 연구에서의 결과를 재현할 수 있었다

주요어: 만성통증, 통각과민, CFA, DREADDs. hM_4D_i, von Frey test, anterior cortex

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