이학석사학위논문

렌티바이러스를 이용한 CRISPR/Cas9의 생체 내 발현 최적화에 관한 연구

Optimization of lentiviral CRISPR/Cas9 *in vivo* expression

2019년 2월

서울대학교 대학원

뇌과학협동과정

고광희

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지도교수 강봉균

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Optimization of lentiviral CRISPR/Cas9 *in vivo* expression

Advisor. Professor Bong-Kiun Kaang

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

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Kwang-Hee Ko

Graduate School of Natural Sciences

Seoul National University

Interdisciplinary Program in Brain Science

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Abstract

Optimization of lentiviral CRISPR/Cas9 *in vivo* expression

Kwanghee Ko

Interdisciplinary Program in Neuroscience

The Graduate School

Seoul National university

Clustered Regularly Interspaced Short Palindromic Repeats /CRISPR-associated 9 (CRISPR/Cas9) system is gaining attention among scientists because of its specificity in genomic sequence recognition and feasibility in gene editing. Although the number of studies using CRISPR/Cas9 system is increasing in the field of neuroscience, most of them were conducted in *in vitro* condition such as primary neuron cultures or organotypic slice cultures. In spite of its versatility, the studies using CRISPR/Cas9 system in more physiological *in vivo* condition have not been realized much so far.

Here, we search for the optimal lentivirus production condition for

the CRISPR/Cas9 system *in vivo* expression. By optimizing the medium for mammalian cell culture, rotor type during ultracentrifugation, DNA vector molar ratio at transfection, and the insert size of transfer vector, we increased the purity and titer of lentiviral solution, and succeeded in producing high titer lentiviral CRISPR/Cas9 that is compatible with electrophysiological experiments and behavioral analyses after *in vivo* expression.

The single guide RNA (sgRNA) construct which targets glutamate ionotropic receptor AMPA type subunit 2 (GluA2) of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) was used to delete GluA2 subunit, and GluA2 knockout was confirmed with western analysis and patch clamp recording experiments. The lentiviral CRISPR/Cas9 *in vivo* expression is going to be useful not only for the general biological study, but also for the biomedical research.

Keyword: lentivirus, CRISPR/Cas9 system, GluA2 subunit, AMPAR, western blot analysis, patch clamp recording

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Introduction

To deliver the gene of interest for successful research and medical therapeutics, an appropriate gene delivery method is required. A number of techniques to deliver gene of interest have been developed, such as non-viral delivery method like lipid mediated transfection or electroporation, and viral delivery method using adeno-associated virus (AAV), lentivirus, or adenovirus (Liu et al., 2017; Yin et al., 2017; Lino et al., 2018). For delivering the gene of interest *in vivo* for a therapeutic purpose, the viral delivery method is more feasible than non-viral method, since naked gene of interest is vulnerable in *in vivo* environments because of the nuclease activity (Yin et al., 2017). This makes it more feasible to use the viral delivery system than non-viral delivery methods for *in vivo* expression. Especially, scientists are interested in delivering the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR), which is regarded as the next generation gene editing system, to the live organisms (Wang et al., 2016; Adli, 2018). CRISPR is an immune system protecting prokaryotes from viruses, using its outstanding DNA sequence recognition ability. CRISPR/Cas9 system attracted scientists, and gained attention as the next generation gene editing technology for biological research or medical therapeutic purposes, because of its specificity in recognizing the target sequence and its feasibility in exploitation. The CRISPR technology progressed substantially, and it is now possible to do experiments using CRISPR/Cas9 system as follows: gene knockout, gene replacement,

transcriptional regulation, epigenome editing, base editing and CRISPR imaging, and so on (Wang et al., 2016; Adli, 2018).

Both lentivirus and AAV have been used to deliver CRISPR/Cas9 system (Liu et al., 2017; Yin et al., 2017; Lino et al., 2018). Lentivirus has an advantage over AAV in that it has a larger DNA cargo capacity than AAV. Whereas packaging capacity of lentivirus is up to 8kb, that of AAV is up to 4.7kb. The fact that the DNA size of SpCas9, which is the most efficient Cas9 protein so far, is 4.2kb, makes packaging CRISPR/Cas9 system using AAV challenging because inclusion of single guide RNA (sgRNA) DNA or fluorescent protein DNA in transfer vector often exceeds AAV cargo capacity limit. To circumvent this problem, scientists keep exploring for the new Cas9 proteins that are small enough to be packaged with sgRNA, and fluorescent protein DNA in AAV. Despite of the efforts so far, the SpCas9 is the most efficient Cas9 protein so far. Discovering the alternatives of SpCas9 protein is still ongoing. Another possible resolution to overcome the limited DNA cargo capacity of AAV is the split CRISPR/Cas9 system. In this case, when the split CRISPR/Cas9 system is expressed simultaneously in one cell, the split CRISPR/Cas9 system are recombined to form a completely functional CRISPR/Cas9 system in the target cell. Although this seems better than previous AAV-CRISPR/Cas9 system, its efficiency may be lower than typical lentiviral CRISPR/Cas9, because the split CRISPR/Cas9 system has to be recombined to complete CRISPR/Cas9 system in the target cells.

The problem of using lentivirus is that it is extremely difficult to

produce high titer virus. Although there have been several publications for producing high titer lentivirus compatible for *in vivo* expression (Tiscornia et al, 2006; Kutner et al, 2009; Ohkawa et al, 2015; Yokose et al, 2017), in case of CRISPR/Cas9 system, *in vivo* expression by lentivirus has not been realized except a few studies (Zheng et al., 2018).

Here, our focus was on optimizing procedures for getting high titer lentiviral CRISPR/Cas9, which is appropriate for *in vivo* expression. We succeeded in producing high titer lentiviral CRISPR/Cas9 that is compatible for electrophysiological or behavioral experiments after *in vivo* expression. We transduced lentiviral CRISPR/Cas9 in mouse hippocampal CA1 to characterize calcium permeable AMPAR, an AMPAR variant that lacks GluA2 subunit (Jia et al., 1996). AMPAR is a heterotetramer or homotetramer composed of diverse combinations of GluA1, GluA2, GluA3, and GluA4 subunits. It is known that majority of AMPAR of hippocampal CA1 pyramidal neurons have GluA2 subunit, which make AMPAR calcium impermeable. Here, we deleted GluA2 subunit with a sgRNA construct that targets GluA2 subunit, generating GluA2 lacking AMPAR, which is calcium permeable. GluA2 knockout was confirmed with western analysis. Inwardly rectified AMPAR current in patch recording in acute brain slices after *in vivo* expression indicated that CRISPR/Cas9 expressing neurons lost GluA2 subunits, which is a signature property of GluA2 lacking AMPAR (Incontro et al., 2014; Park et al., 2015).

Materials and Methods

Plasmid construct

The following oligonucleotide sequences were used to generate single guide RNA (sgRNA) for GluA2 knockout: forward (5' to 3') CACCGctaacagcatacagataggt; reverse (5' to 3') AAACacctatctgtatgctgttagC (Incontro et al., 2014). These were annealed and ligated into lentiCRISPR v2 GFP (Feldser lab) using Esp3I. The EF1α core promoter was exchanged to PCR-amplified CaMKIIα promoter at the NheI-AgeI restriction sites of lentiCRISPR v2 GFP vector. PCR-amplified tdTomato replaced GFP.

Lentivirus production

Lenti-X 293T cells were maintained in DMEM (with additional 10% FBS, 2mM L-Glutamine, 0.1mM MEM Non-Essential Amino Acids, 1% 100 x Penicillin/Streptomycin). Lentivirus was produced by transfecting Lenti-X 293T cells with following plasmids: pMD2.G, psPAX2, and lentiCRISPR v2. After 8~12hours transfection, the media was replaced with UltraCULTURE™ (with additional 4mML-Glutamine, 2mM Glutamax-I, 0.1mM MEM Non-Essential Amino Acids, 1mM Sodium Pyruvate, 1% 100x Penicillin/Streptomycin). The supernatant was harvested two times (2day

post-transfection and 3day post-transfection), centrifuged (500g, 10minutes, room temperature), and filter-sterilized with a 0.45μm filter (Merck, Cat. no SLHV033RS). Filtered solution was pooled and ultracentrifuged (20,000rpm, 2 hours, 4°C) using SW32Ti rotor (Beckman Coulter) with 3ml of 20% sucrose cushion. The lentivirus pellet was resuspended in Dulbecco's Phosphate Buffered Saline (DPBS).

Lentivirus titration

Lentiviral titer was measured in two ways. One was titrating with qPCR-based lentivirus titration kit (abm, Cat # LV900), following the manual. The SYBR Green II (Takara) was used as the 2X qPCR Mastermix. The other method was treating 1ul of serial diluted lentivirus to 10^5 HEK293T cells. The fluorescence ratio was used for measuring the range of infectious lentivirus titer as reported previously (Tiscornia et al, 2006).

Neuronal culture

Hippocampal cultures were prepared from rat E18 embryos. For western blot, $3.0x10^5$ cells were plated per well in a 6-well plate. The wells were coated with poly-D-lysine overnight before plating and neurons were plated in plating media (10% FBS in MEM) overnight. The media was exchanged to maintenance media (neurobasal media with additional B-27).

Western blot(WB)

For GluA2 knockout confirmation, lentiviral CRISPR/Cas9 treated neurons were lysed with RIPA buffer and quantified with BCA protein assay. The protein samples were loaded onto 12% SDS/PAGE and transferred to ECL membrane. The membrane was blocked with 5% skim milk (in TBST). Mouse anti-GluA2 in 5% skim milk solution was treated for overnight with gentle rocking. Primary antibody was removed, rinsed with TBST, and treated with donkey anti-mouse IgG-HRP conjugate for in 5% skim milk solution. Blots were developed using either Immobilon Western Chemiluminescent HRP substrate or Immobilon Classico Western HRP substrate and imaged using ChemiDoc.

Animals

Adult male mice (C57BL/6J or C57BL/6N) were used for entire study of *in vivo* expression. Mice were bred under standard conditions. All procedures were conducted under sterile conditions and approved by the International Animal Care and Use Committee (IACUC).

Stereotaxic surgery

All surgical processes were conducted under sterile conditions and approved by the International Animal Care and Use Committee (IACUC) of Seoul National University. Mice (male, 8~10 weeks of age) were

anaesthetized by intraperitoneal injection of ketamine/xylazine. The anaesthetized mice were immobilized on a stereotaxic apparatus (Stoelting) and the lentiviral medium (0.5μl) was injected using a syringe with 31gauge needle (Hamilton) at a 0.1μl/min rate into target regions. Stereotaxic coordinates for hippocampal CA1: (AP: −1.7mm, ML: ±1.5mm, DV: −1.6mm below the skull surface), (AP: −2.2mm, ML: ±2.0mm, DV: −1.6mm below the skull surface).

Brain preparation and imaging

After 3~7 weeks of expression, the brain was removed, fixed with 4% paraformaldehyde (PFA) in PBS for overnight, and dehydrated with 30% sucrose in PBS for 2 days at 4°C. After freezing, brain slices (50μm) were prepared with a cryostat and mounted on a glass slide with 50% glycerol in PBS. Hippocampal CA1 regions of the brain slices were imaged by Nikon fluorescence microscope or ZEISS confocal microscope.

Hippocampal slice preparation and whole cell patch recording

After 3~6 weeks of lentiviral CRISPR/Cas9 injection, hippocampal slices were prepared. The animals were sacrificed and the brain was removed. The removed brain was put in cold solution that contained 124mM NaCl, 3mM KCl, 26mM $NaHCO_3$, 1.25mM NaH_2PO_4, 10mM $MgSO_4$,

10mM D-glucose and 1mM $CaCl_2$, saturated with 95% O_2 and 5% CO_2. Hippocampus was sliced with vibratome in slicing solution. Slices were recovered at 32-34℃ for 30 minutes. Slices were kept at 26-28℃ for at least 1 hour before recordings were begun.

Whole cell recording was made at 32℃ with artificial cerebrospinal fluid containing 50μM picrotoxin and 20uM (+)-bicucullin. The whole-cell solution was composed of 8mM NaCl, 130mM CsMeSO3, 10mM HEPES, 0.5mM EGTA, 4mM Mg-ATP, 0.3mM Na3-GTP, 5mM QX-314 and 0.1mM spermine.

Rectification index (RI) measurement

AMPAR currents were recorded using a NMDAR antagonist (D-AP5; 100 μM) with a glycine-site antagonist (L-689,560; 5 μM). Neurons were depolarized to + 40 mV for 100 s. Next, neurons were depolarized to 0 mV for 50 s. The rectification index was obtained by dividing the slope from 0 to + 40 mV, by the slope from -70 to 0 mV. RIs were compared between lentiviral CRISPR/Cas9 infected neurons and non-infected neurons.

Result

Initial trials of lentiviral CRISPR/Cas9 *in vivo* expression (collaborated with Ji-il Kim, Daehee Han)

We initially produced lentiviral CRISPR/Cas9 following our previous protocol. To produce lentiviral CRISPR/Cas9, the pLenti-CRISPR v2 GFP was co-transfected with pMD2.G and psPAX2. After 6~8 hours of transfection, the medium was changed to DMEM (FBS 10%). 3 days later, the lentivirus containing-supernatant was harvested, filter-sterilized, and concentrated using 70Ti rotor (Beckman Coulter) with 3ml of 20% sucrose cushion. The titration was done using qPCR based-lentivirus titration kit (abm Cat # LV900), and the resultant titer was 2.0×10^9IU/ml.

Next, we injected 0.3μl of LV-U6-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-GFP into the hippocampal CA1 by stereotaxic surgery. After 3weeks and 5weeks of expression, mice were sacrificed, brains were removed, and imaged with confocal microscope (Fig. 1A, B).

The GFP signal in the confocal microscopy was too weak (Fig. 1A), which was not consistent with the previous studies that used lentivirus whose titer is above 10^9IU/ml (Noriaki et al, 2015; Yokose et al, 2017). This result indicated that the expression efficiency is too low and not feasible for patch clamp recording. Furthermore, after 5weeks of expression, there was no GFP signal visible, which implied neuronal death (Fig. 1B). It was more evident that the lentiviral solution induces neuronal

death when the lentivirus was co-injected with AAV-CaMKIIα-mCherry, because mCherry signal was absent in the region of the injection sites (Fig. 1C).

We also injected LV-U6-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-GFP for patch recording experiments. After more than 3 weeks of expression, the mouse was sacrificed and patch clamp recordings were conducted. The results of the patch clamp recording also indicated that this lentiviral CRISPR/Cas9 is not appropriate for electrophysiological study after *in vivo* expression, because many neurons were dead and no fluorescence could be detected (data not shown).

There were several ways to circumvent neuronal death problem. First, it is possible to dilute the virus solution. However, it was not compatible for this lentiviral CRISPR/Cas9, because of its low titer. It was evident that the weak fluorescent signal will get even weaker after dilution. Secondly, it is possible to inject smaller volume of lentiviral CRISPR/Cas9. This was not feasible because our ultimate goal was expressing CRISPR/Cas9 for electrophysiological experiments, which need 350~400μm slices. Smaller volume may express CRISPR/Cas9 in extremely restricted area, making electrophysiological experiments unfeasible. Lastly, expressing lentivirus for short period, such as 1 or 2 weeks was another way to reduce neuronal death problem. This was also not compatible with our goal, which was deleting GluA2 subunit. It takes time not only for the genomic GluA2 to be deleted, but also for the remaining GluA2 proteins to be degraded after genomic GluA2 knockout.

As Incontro et al. deleted GluA2 subunit in organotypic slice culture by expressing CRISPR/Cas9 for 2 weeks (Incontro et al., 2014), it was not likely that expressing 1 or 2 weeks is enough for GluA2 knockout. The weak GFP signal and neuronal death in these serial results indicated that the both the purity and titer of lentiviral CRISPR/Cas9 have to be improved for lentiviral CRISPR/Cas9 *in vivo* expression. At first, we searched for the condition that can increase the lentiviral purity.

Searching for the condition for producing higher-purity lentivirus

To increase purity, we excluded the FBS from virus producing medium—the exchanged media after transfection, as it is reported that the presence of FBS in lentivirus solution can induce immune response in mouse brain, resulting in low gene expression efficiency or neuronal death (Scherr et al., 2002; Baekelandt et al., 2003; Merten et al., 2016). Next, we treated benzonase to digest the unpackaged RNA in the virus containing solution before ultracentrifugation, because the lentivirus produced for gene therapy use this method to increase purity (Bandeira et al., 2012). Moreover, we exchanged the rotor type from fixed angle rotor to swing rotor, because it is reported that swing rotor is more appropriate than fixed angle rotor for purification using sucrose cushion (Castaneda et al., 1971). Also, all of the published protocols used the swing rotor during ultracentrifugation (Tiscornia et al., 2006; Kutner et al., 2009). Lastly, after

lentiviral pellet resuspension, we purified the lentiviral solution using Amicon Ultra-15 Centrifugal Filter (100,000NMWL) to purify the lentiviral solution.

We co-transfected the pLenti-CRISPR v2 GFP, pMD2.G, psPAX2 in Lenti-X 293T cells. 6~8 hours after transfection, the medium was exchanged to the DMEM. 3 days later the lentivirus containing solution was concentrated with SW32Ti rotor (20,000rpm, 2h, 4℃), and the lentiviral pellet was resuspended in DPBS. This lentiviral solution was purified using Amicon Ultra-15 Centrifugal Filter (100,000NMWL). The titer was measured with qPCR-based titration kit (abm Cat # LV900) and the resultant titer was 1.89×10^7IU/ml. The decrease of titer may have been originated by exclusion of FBS, which is critical for mammalian cell condition and growth, or use of Amicon Ultra-15 Centrifugal Filter (100,000NMWL), as the lentiviral particles may have stuck on the membrane. Also, as unpackaged RNA can increase the titer, digesting with these RNA with benzonase may have reduced the titer.

To test the *in vivo* expression pattern, 1μl of the lentivirus solution was injected into the mouse hippocampal CA1. After 5 weeks of expression, the brain was removed and imaged. Promisingly, the hippocampal CA1 pyramidal neurons showed no neuronal death, even we injected large volume of lentivirus and expressed for 5 weeks. The patch clamp recording result was also promising as it showed no neuronal death. However, the GFP signal was still too weak and barely visible even in the powerful confocal imaging, and the number of GFP positive neurons were too small

(Fig. 2). These results indicated that although the purity of lentiviral solution was improved, the titer has to be significantly increased.

Searching for the condition for producing higher-titer lentivirus

As the FBS improves the mammalian cell condition, it helps to increase the lentiviral CRISPR/Cas9 titer. However, as the remaining FBS in lentiviral solution can reduce the gene expression efficiency or induce neuronal death (Scherr et al., 2002; Baekelandt et al., 2003), the process finding the most appropriate virus production medium without FBS was required. We tried to find out the most appropriate medium for high titer lentivirus production, as there are many kinds of medium reported so far (Yokose et al., 2017; Zheng et al., 2018). This time, we did not use the Amicon Ultra-15 Centrifugal Filter (100,000NMWL) and benzonase, as most of the protocols we referred to did not use them. We packaged the pLenti-U6-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-tdTomato, expecting that tdTomato will be more clearly visible than GFP.

pLenti-U6-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-tdTomato, pMD2.G, and psPAX2 were transfected. 12 hours after transfection, medium was exchanged to candidate virus production medium. Each candidate virus production medium was as follows: DMEM (L-glutamine 4mM, MEM-NEAA 0.1mM, glutamax I 2mM, sodium pyruvate 1mM, 1% 100x Penicillin/Streptomycin), DMEM (FBS 10%, L-glutamine 4mM, MEM-

NEAA 0.1mM, glutamax I 2mM, sodium pyruvate 1mM, 1% 100x Penicillin/Streptomycin), UltraCULTURE (L-glutamine 4mM, MEM-NEAA 0.1mM, glutamax I 2mM, sodium pyruvate 1mM, Penicillin/Streptomycin). 3 days after transfection, lentivirus containing-supernatant was harvested, filter-sterilized, and concentrated with SW32Ti rotor (20,000rpm, 2h, 4℃). The lentivirus produced from each medium was titrated using qPCR-based lentivirus titration kit (abm Cat # LV900). This time, the lentivirus produced from each medium was also treated to mammalian cells to see which condition resulted in more infectious lentivirus.

The titer measure by titration kit was as follows: $2.09x10^9$IU/ml for DMEM (FBS 10%, L-glutamine 4mM, MEM-NEAA 0.1mM, glutamax I 2mM, sodium pyruvate 1mM, 1% 100x Penicillin/Streptomycin), $1.01x10^9$IU/ml for DMEM (L-glutamine 4mM, MEM-NEAA 0.1mM, glutamax I 2mM, sodium pyruvate 1mM, 1% 100x Penicillin/Streptomycin), and $9.44x10^8$IU/ml for UltraCULTURE™ (L-glutamine 4mM, MEM-NEAA 0.1mM, glutamax I 2mM, sodium pyruvate 1mM, 100x Penicillin/Streptomycin). Surprisingly, the amount of infectious lentiviral CRISPR/Cas9 was largest in the UltraCULTURE (L-glutamine 4mM, MEM-NEAA 0.1mM, glutamax I 2mM, sodium pyruvate 1mM, 1% 100x Penicillin/Streptomycin) and lowest in the DMEM (L-glutamine 4mM, MEM-NEAA 0.1mM, glutamax I 2mM, sodium pyruvate 1mM, 1% 100x Penicillin/Streptomycin) (Fig. 3). This indicated that the qPCR based-lentivirus titration method may not be reliable. We decided to use the UltraCULTURE™ (L- glutamine 4mM, MEM-NEAA 0.1mM, glutamax I 2mM, sodium pyruvate 1mM, 1% 100x

Penicillin/Streptomycin), which gave the highest amount of infectious lentivirus, for high titer lentiviral CRISPR/Cas9 production.

High titer LV-U6-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-tdTomato production

To produce high titer LV-U6-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-tdTomato, we transfected twelve 150 pi plates. 12 hours after transfection, we exchanged medium to UltraCULTURE™ (L-glutamine 4mM, MEM-NEAA 0.1mM, glutamax I 2mM, sodium pyruvate 1mM, 1% 100x Penicillin/Streptomycin). 3 days post-transfection, concentration and titration was conducted. The titer measured with qPCR-based lentivirus titration kit was $1.99x10^{10}$IU/ml. The titer measured by treating lentivirus to mammalian cells was up to $5x10^{8}$TU/ml. This again clarified that the qPCR-based lentivirus titration method is not reliable, at least for lentiviral CRISPR/Cas9.

1μl of LV-U6-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-tdTomato was injected into the mouse hippocampal CA1 by stereotaxic surgery. LV-U6-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-tdTomato was expressed at least three weeks and then sacrificed for fluorescence imaging, confocal imaging, and patch recording experiments. As reported previously, the lentiviral expression was sparse (Fig. 4B,D) (Wu et al., 2017). Also, it was promising that the tdTomato expressing area was very long in anterior-posterior axis, up to 1200μm. Furthermore, the level of neuronal death was

significantly reduced compared to before. These enhanced expression level indicated that this condition was compatible with patch recording experiments.

It is notable that lentiviral expression was also abundant in dentate gyrus (DG), which was not observed when using AAV (Fig. 4C,E). This may be due to the fact that injection site was lower than pyramidal layer, and lentivirus is much heavier than AAV, making lentivirus sink to the DG. These results indicated that the injection site may have to be raised to above the pyramidal layer.

Improvements for higher titer

Although LV-U6-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-GFP whose titer was up to $5x10^{8}$TU/ml provided an excellent environment where GluA2 deleted neurons were comparable with adjacent wild type neurons due to lentiviral sparse expression (Wu et al., 2017), this may not be appropriate for behavioral analyses because they usually require most of the neurons near the injection sites to be infected. There was a study suggesting that lentiviral CRISPR/Cas9 whose titer is 10^{9}~10^{10}TU/ml is appropriate for the behavioral experiments after *in vivo* expression (Fricano-Kugler et al., 2016). We sought for the condition that can result in this level of lentiviral CRISPR/Cas9 titer.

It is known that the insert size of transfer vector affects the lentiviral titer (Kumar et al., 2001). Lentivirus cargo capacity limit is about

8kb, and insert size larger than this limit negatively affects lentivirus titer. Also, it is thought that as the insert size increases for 1kb~2kb, the lentiviral titer decreases to 10%. As the insert size of LV-U6-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-tdTomato was about 8.7kb, we changed the fluorescent protein into the GFP construct (Fig. 5B). By exchanging tdTomato to GFP resulted in 720bp reduction of transfer vector insert size, and the insert size of pLenti-U6-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-GFP was about 8kb.

Vesicular stomatitis virus glycoprotein (VSVG) of lentivirus is the most critical factor for the lentivirus to be infectious. The shortage of VSVG amount during transfection may have been the cause of low lentivirus titer. We investigated the molar ratio of VSVG-encoding envelope vector, packaging vector, and transfer vector of other protocols or publications (Table 1). Although there were some similar ratios in three vector molar ratio at transfection, many laboratories used higher envelope vector ratio than ours. We decided to use DNA ratio of Luikart's laboratory protocol, who succeeded in producing lentiviral CRISPR/Cas9 whose titer was 10^9~10^{10}TU/ml and transducing in mouse brain (Fricano-Kugler et al., 2016).

The concentration was done the same way as above. We did not used the qPCR-based lentivirus titration kit anymore, as it seemed that it is not a reliable method to measure the amount of infectious lentiviral CRISPR/Cas9. The resultant infectious titer was ~5x10^9TU/ml. 0.5 μl of LV-U6-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-GFP was injected into the

mouse hippocampal CA1 by stereotaxic surgery. LV-U6-CRISPR_GluA2-CamKIIα-SpCas9-P2A-GFP was expressed for four weeks and then sacrificed for fluorescence imaging and confocal imaging. The fluorescence image and confocal image show that most of the hippocampal CA1 is GFP-positive (Fig. 5C,D). This indicates that this range of lentivirus titer may be appropriate for behavioral analyses, which need whole expression of target regions. Also, it was remarkable that the expression range was narrower than that of AAV. As we tried to target the site just above the pyramidal layer to avoid lentiviral expression in DG, there was no observable expression in DG. This again implies that different dorsal-ventral axis coordination of stereotaxic surgery has to be used for AAV and lentivirus.

To verify what dilution factor has to be used to label hippocampal CA1 neurons sparsely, 0.5μl of LV-U6-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-GFP that was diluted 2 times and 5 times, and injected in hippocampal CA1. The GFP expression was examined almost all of the neurons in the injected site of lentiviral CRISPR/Cas9 that is diluted 2 times (Fig. 6A,D). The expression pattern of lentiviral CRISPR/Cas9 that is diluted 5 times was sparser (Fig. 6B,E). However, it is not sure whether the sparse expression originated from 5 times dilution, because the lentiviral expression was also abundant in DG this time (Fig 6C,F). The expression in DG may have reduced the lentiviral expression in CA1. It has to be tested whether what dilution factor gives sparse expression.

GluA2 knockout verification (collaborated with Dr. Pojeong Park, Sanghyun Ye)

To test whether lentiviral CRISPR/Cas9 really deleted GluA2 subunit, we infected primary hippocampal neurons and conducted western blot analysis, and patch clamp recording. Western blot analysis showed that the GluA2 protein level decreases as the amount of lentiviral CRISPR/Cas9 treated increases. (Fig. 7). These biochemical results in primary neuron culture indicated that the lentiviral CRISPR/Cas9 indeed deleted GluA2 subunit of infected neurons, confirming the function of our sgRNA construct.

The ultimate goal of this lentiviral CRISPR/Cas9 in this study was to delete GluA2 subunit of adult mice, generating endogenous calcium permeable AMPAR, which is an AMPAR variant that lacks GluA2 subunit. GluA2 lacking AMPAR has its signature inwardly rectifying AMPAR currents, and this index is used frequently for identifying GluA2 lacking AMPAR. In the acute slices of the mouse that had been injected LV-U6-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-tdTomato or LV-U6-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-GFP and expressed more than 5 weeks, the fluorescence signal was significantly improved than before (Fig. 8A), and the tdTomato-positive or GFP-positive pyramidal neurons showed inwardly rectifying AMPAR current (Fig 8B), which means that the GluA2 subunits of lentiviral CRISPR/Cas9 infected neurons were really deleted. The tdTomato-negative or GFP-negative pyramidal neurons showed AMPAR current of AMPAR variants that possess GluA2 subunits. These electrophysiological data confirming GluA2 subunit knockout

indicated that the lentiviral CRISPR/Cas9 *in vivo* expression was successful.

Production of higher titer LV-FUW-rtTA and LV-tetO-Ngn2-P2A-EGFP-T2A-Puro

As we succeeded in producing high titer lentiviral CRISPR/Cas9, we considered application of high titer lentivirus production for other purposes. As we were using lentivirus for neuronal differentiation from stem cells following Sudhof's protocol (Zhang et al., 2013), we applied our new lentivirus production protocol. Before the lentivirus protocol revision, we used DMEM (10%) for mammalian cell culture and fixed angle rotor (70Ti) for ultracentrifugation. After the revision, we used DMEM (FBS 10%, L-glutamine 4mM, MEM-NEAA 0.1mM, 1% 100x Penicillin/Streptomycin) for mammalian cell culture and the swing rotor (SW32Ti) for ultracentrifugation. The lentivirus was treated to mammalian cells, and fluorescence ratio was examined after treating doxycycline. The lentivirus produced with revised protocol gave more fluorescence than before, as 1μl treatment was enough to show almost 100% fluorescence ratio, whereas even 8μl was not enough to show 50% fluorescence ratio for the lentivirus produced by the protocol before revision. This increase is thought to be originated from additional factors, which improve the nutrition conditions of medium, resulting in better mammalian cell conditions. Higher titer of lentivirus is expected if UltraCULTURE^{TM} (L-glutamine 4mM, MEM-NEAA 0.1mM, glutamax I 2mM, sodium pyruvate 1mM, 1% 100x

Penicillin/Streptomycin) is used.

Figures

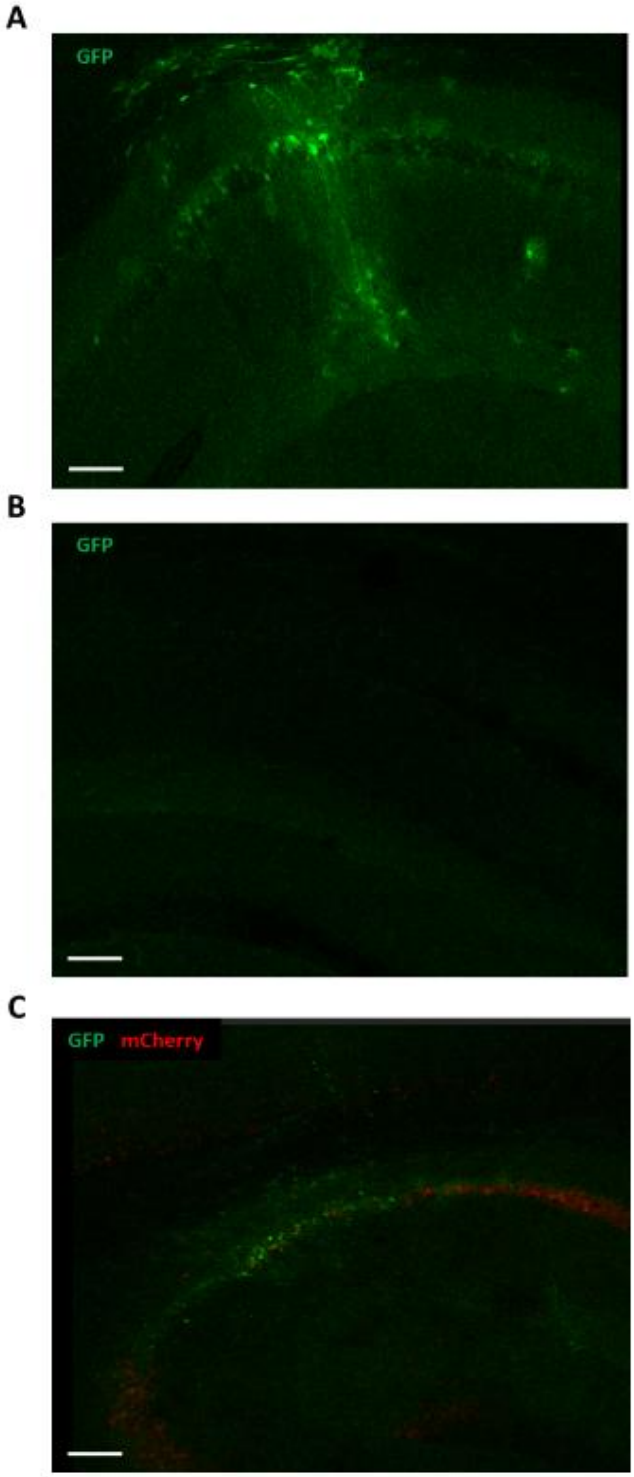

A
GFP
B
GFP
C
GFP
mCherry

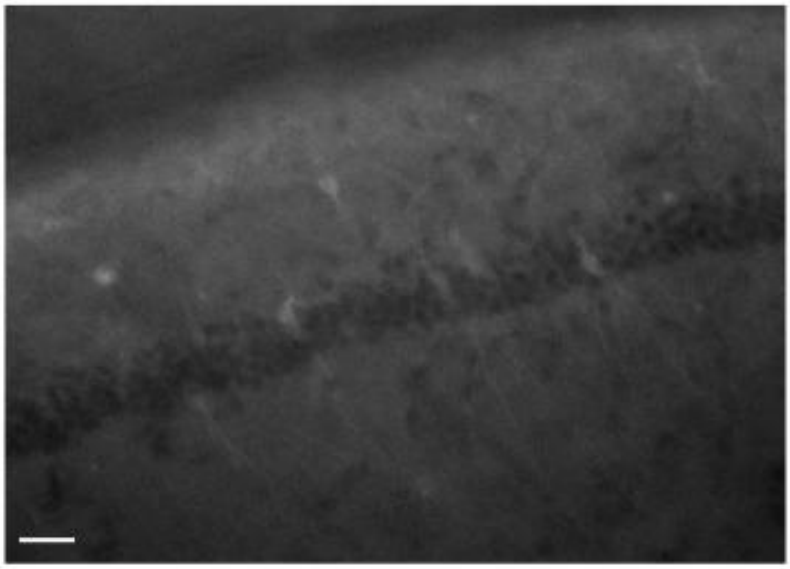
Figure 1 Initial trials of lentiviral CRISPR/Cas9 system in hippocampal CA1 *in vivo* expression (collaborated with Ji-il Kim, Daehee Han).

A. In vivo expression of LV-U6-CRISPR_GluA2-CamKIIα-SpCas9-P2A-GFP for 3 weeks (scale bar, 100μm).

B. In vivo expression of LV-U6-CRISPR_GluA2-CamKIIα-SpCas9-P2A-GFP for 5 weeks (scale bar, 100μm).

C. In vivo expression of LV-U6-CRISPR_GluA2-CamKIIα-SpCas9-P2A-GFP and AAV-CamKIIα-mCherry for 4 weeks (scale bar, 100μm).

A



B

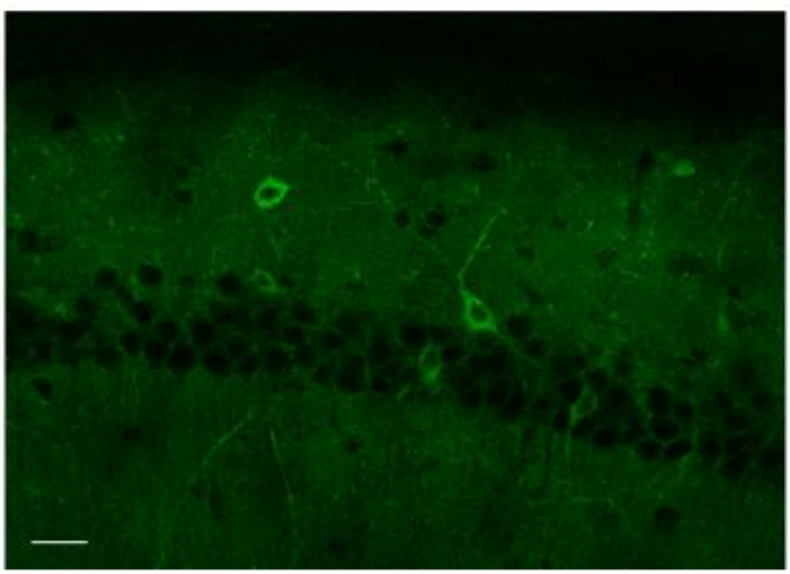
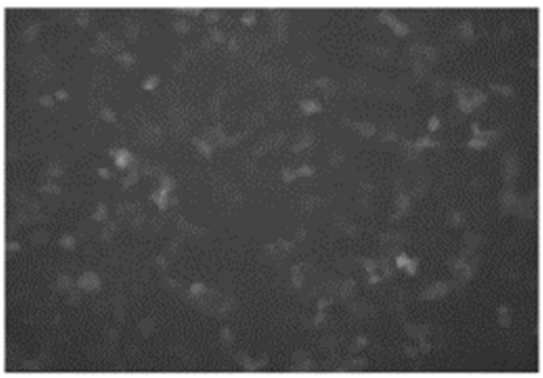


Figure 2 lentiviral CRISPR/Cas9 *in vivo* expression after modulating the factors that can affect purity.

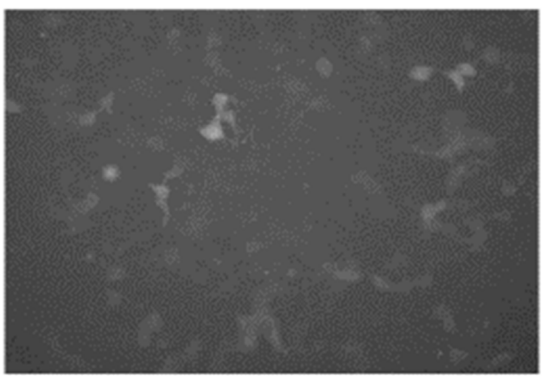
A. Fluorescence imaging of in vivo expression of LV-U6-CRISPR_GluA2-CamKIIα-SpCas9-P2A-GFP for 5 weeks (scale bar, 50μm)..

B. Confocal imaging of in vivo expression of LV-U6-CRISPR_GluA2-CamKIIα-SpCas9-P2A-GFP for 5 weeks (scale bar, 20μm)..

A



B



C

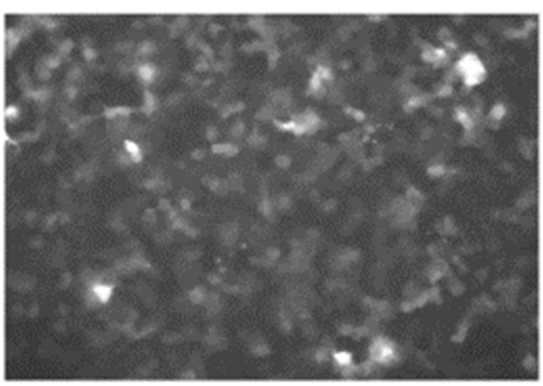


Figure 3 Finding the optimal medium that results in the highest lentiviral CRISPR/Cas9 titer.

A. DMEM (L-glutamine 4mM, MEM NEAA 0.1mM, glutamax I 2mM, sodium pyruvate 1mM, Penicillin/Streptomycin)

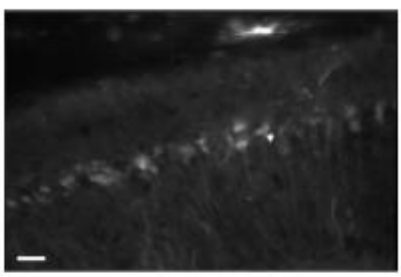
B. DMEM (FBS 10%, L-glutamine 4mM, MEM NEAA 0.1mM, glutamax I 2mM, sodium pyruvate 1mM, Penicillin/Streptomycin)

C. Ultraculture (L-glutamine 4mM, MEM NEAA 0.1mM, glutamax I 2mM, sodium pyruvate 1mM, Penicillin/Streptomycin)

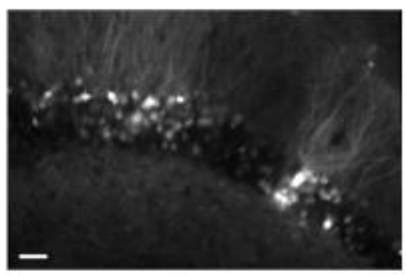
A


Ultraculture
(factors)
X 12
concentration

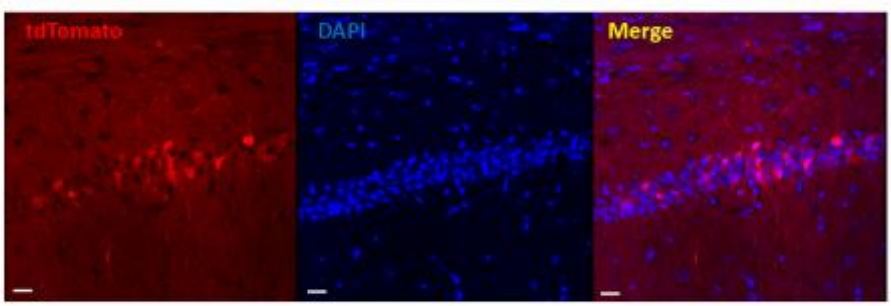
B



C



D


tdTomato
DAPI
Merge

E

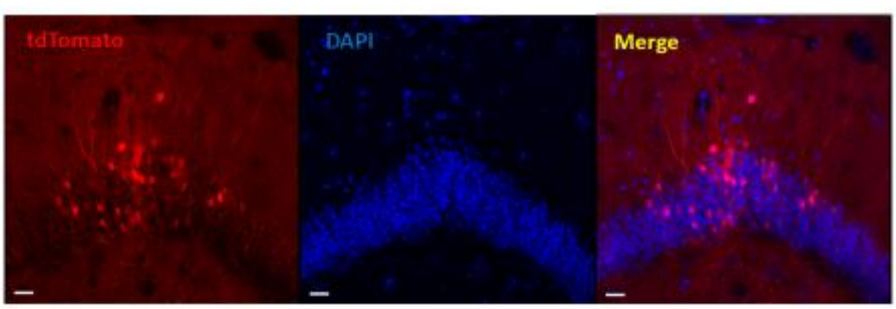

tdTomato
DAPI
Merge

Figure 4 *In vivo* expression of LV-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-tdTomato

A. Scheme of high titer LV-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-tdTomato production

B. Fluorescence image of hippocampal CA1 after *in vivo* expression of LV-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-tdTomato (scale bar, 50μm).

C. Fluorescence image of hippocampal DG after *in vivo* expression of LV-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-tdTomato (scale bar, 50μm).

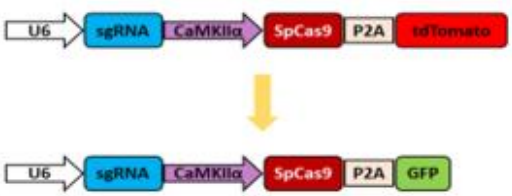
D. Confocal image of hippocampal CA1 after *in vivo* expression of LV-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-tdTomato (scale bar, 20μm).

E. Confocal image of hippocampal DG after *in vivo* expression of LV-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-tdTomato (scale bar, 20μm).

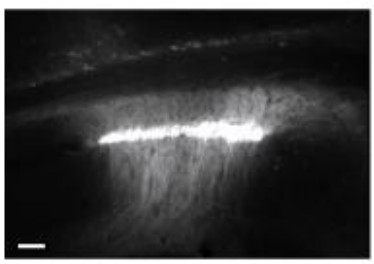
A


Ultraculture
(factors)
X 4
concentration

B


U6
sgRNA
CaMKIIα
SpCas9
P2A
tdTomato
U6
sgRNA
CaMKIIα
SpCas9
P2A
GFP

C



D

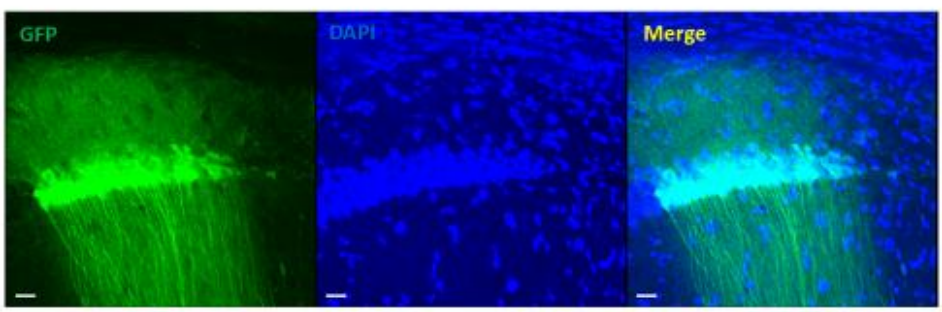

GFP
DAPI
Merge

Figure 5 *In vivo* expression of LV-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-GFP for 4 weeks without dilution

A. Scheme of high titer LV-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-GFP production

B. Changing fluorescence protein from tdTomato to GFP to reduce the insert size of transfer vector

C. Fluorescence image of hippocampal CA1 after *in vivo* expression of LV-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-GFP (scale bar, 50μm).

D. Confocal image of hippocampal CA1 after *in vivo* expression of LV-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-GFP (scale bar, 20μm).

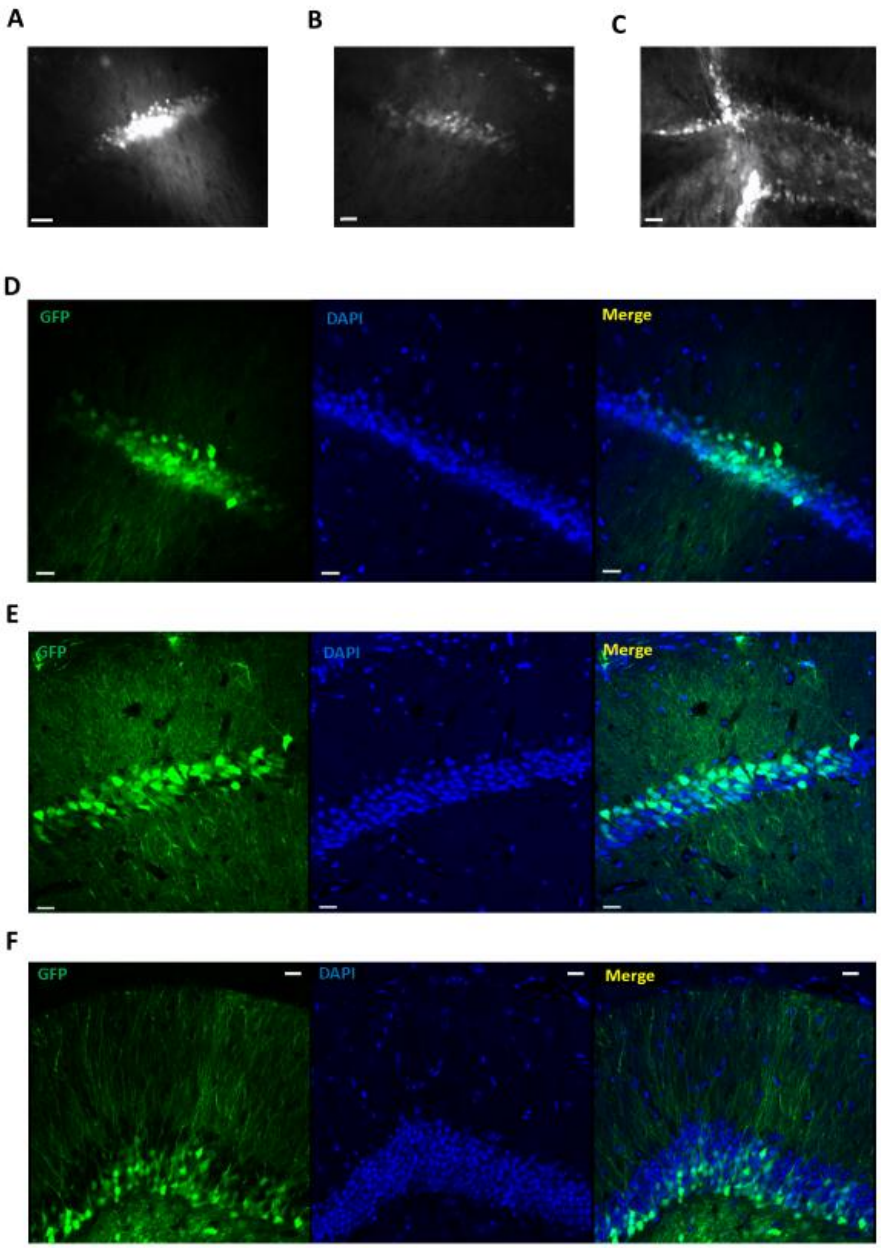

A
B
C
D
GFP
DAPI
Merge
E
GFP
DAPI
Merge
F
GFP
DAPI
Merge

Figure 6 *In vivo* expression of LV-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-GFP for 7 weeks with dilution

A. Fluorescence image of hippocampal CA1 after *in vivo* expression of LV-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-GFP (diluted 2 times, scale bar, 50μm).

B. Fluorescence image of hippocampal CA1 after *in vivo* expression of LV-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-GFP (diluted 5 times, scale bar, 50μm).

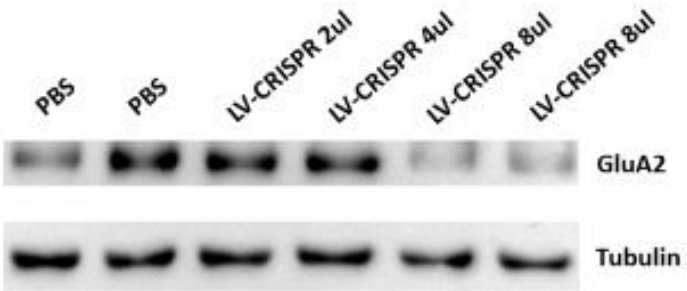
C. Fluorescence image of hippocampal DG after *in vivo* expression of LV-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-GFP (diluted 5 times, scale bar, 50μm).

D. Confocal image of hippocampal CA1 after *in vivo* expression of LV-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-GFP (diluted 2 times, scale bar, 20μm).

E. Confocal image of hippocampal CA1 after *in vivo* expression of LV-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-GFP (diluted 5 times, scale bar, 20μm)

F. Confocal image of hippocampal DG after *in vivo* expression of LV-CRISPR_GRIA2-CamKIIα-SpCas9-P2A-GFP (diluted 5 times, scale bar, 20μm)

A


PBS
PBS
LV-CRISPR 2ul
LV-CRISPR 4ul
LV-CRISPR 8ul
LV-CRISPR 8ul
GluA2
Tubulin

B

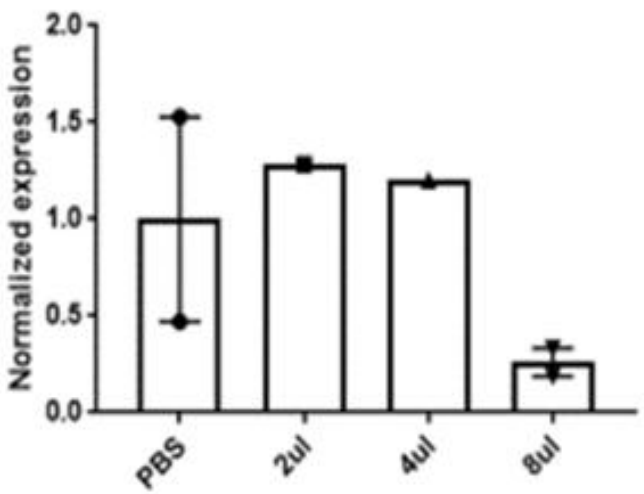
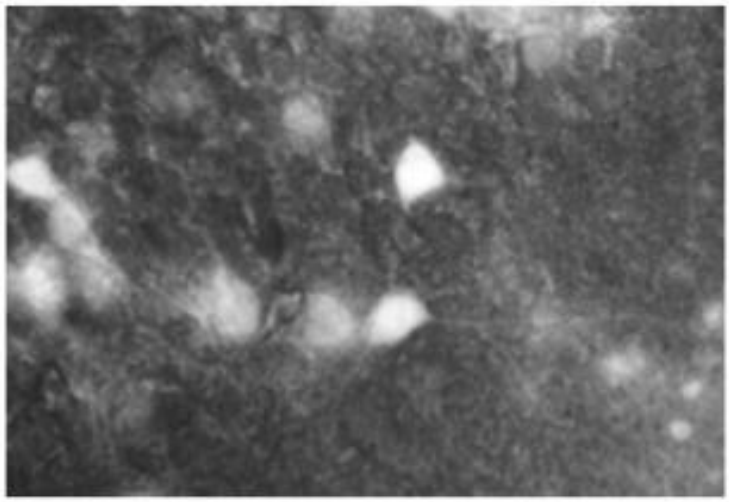

Normalized expression
2.0
1.5
1.0
0.5
0.0
PBS
2ul
4ul
8ul

Figure 7 Western analysis after lentiviral CRISPR/Cas9 system expression in primary neuron culture (collaborated with Sanghyun Ye)

A. Western analysis shows that GluA2 protein level decreases as the amount of lentiviral CRISPR/Cas9 increases.

B. Normalized level of GluA2 (PBS, n=2; 2μl, n=1; 4μl, n=1; 8μl, n=2).

A



B

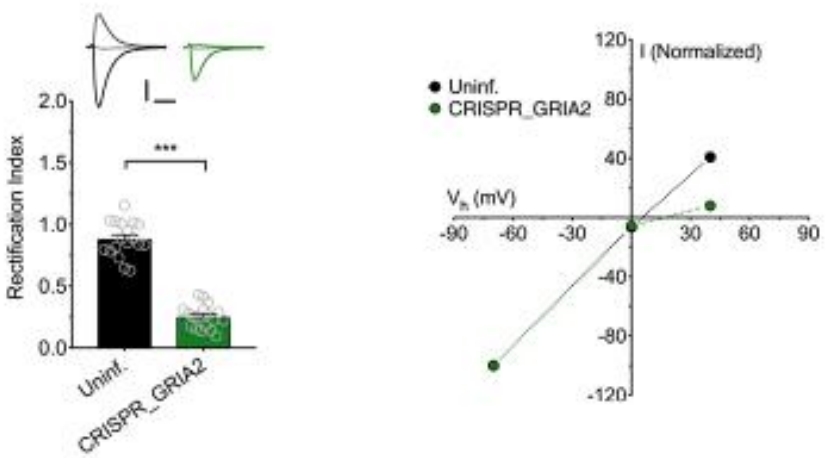
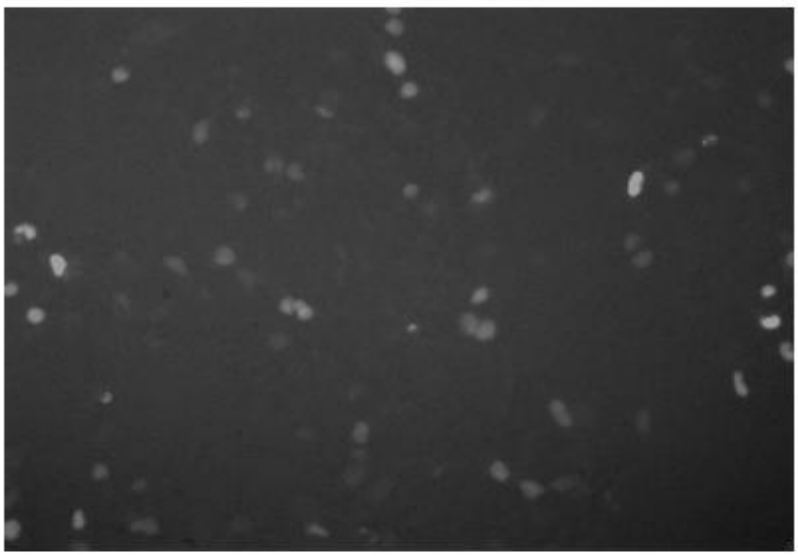

Rectification Index
2.0
1.5
1.0
0.5
0.0
***
Uninf.
CRISPR_GRIA2
I (Normalized)
120
80
40
-40
-80
-120
Uninf.
CRISPR_GRIA2
V_h (mV)
-90
-60
-30
30
60
90

Figure 8 Quantification for rectification index of the isolated AMPAR currents and the corresponding current-voltage relationship. Scale bars: 100 pA and 10 ms. (collaborated with Dr. Pojeong Park)

A. Fluorescence imaging during patch clamp recording

B. Dual whole-cell recordings were made for the CRISPR_GRIA2 expressing and neighboring uninfected (Uninf.) neurons (uninfected, n=17; infected, n=18; unpaired *t*-test; $p < 0.001$).

A



B

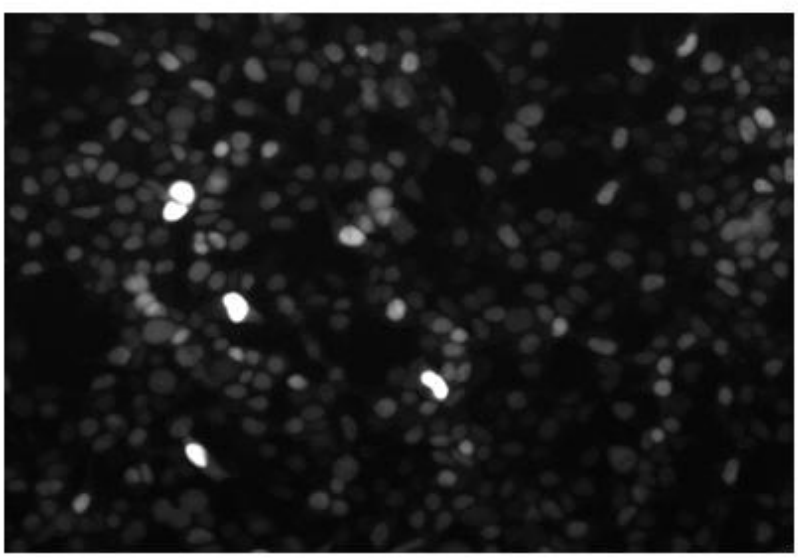


Figure 9 Production of higher titer LV-FUW-rtTA and LV-TetO-Ngn2-P2A-EGFP-T2A-Puro

A. Treatment of 8μl lentivirus produced with DMEM (FBS10%)

B. Treatment of 1μl lentivirus produced with DMEM (FBS 10%, MEM NEAA, L-Glutamine, 1% 100x Penicillin/Streptomycin)

Table 1 Vector molar ratio of different protocols

	Envelope vector	Packaging vector	Transfer vector
Kaang	0.57068	0.620854	1
Inokuchi	1.246659	0.81638/1.736077	1
Deisseroth	1.225333	0.915575	1
Boyden	0.595921	0.743455	1
Verma	0.60318	0.729991/0.25872	1
Kutner	0.557899	0.676438	1
Luikart	1.053864	0.84882	1

Table 2 The plate area used for different protocol

	plate area	plate number	total area
Kaang	175	4	700
Inokuchi	225	4	900
Deisseroth	500	4	2000
Boyden	175	4	700
Verma	175	12	2100
Kutner	150	12	1800
Luikart	65	2	130

Discussion

The CRISPR technology is getting much attention these days and thousands of publications about CRISPR technology are being published. There are several viral methods to deliver CRISPR/Cas9 system *in vivo*, such as AAV and lentivirus. Lentiviral CRISPR/Cas9 system has an advantage over AAV for several reasons. Unlike the AAV which infects almost all neurons near the injection sites, lentivirus has been reported to express sparsely. This makes lentiviral CRISPR/Cas9 system appropriate to compare target gene-deleted phenotypes with nearby non-infected wildtype neurons. Although there is a need for control lentiviral CRISPR/Cas9 packaged with scrambled sgRNA, which does not target any gene, it is a very useful tool to compare knockout phenotype and wildtype phenotype in one mice. Furthermore, lentivirus has larger DNA cargo capacity than AAV, and this can be more advantageous because it is impossible to package a DNA construct which possess all of sgRNA promoter, sgRNA, SpCas9 promoter, SpCas9, and reporter gene, in AAV. Although scientists are looking for other alternative Cas9 protein that is small enough to be packaged in AAV with other essential CRISPR/Cas9 components, SpCas9 is the most efficient Cas9 protein so far and most frequently used.

There were some notable lentiviral expression pattern compared to AAV. In this study, we found that lentiviral expression is typically narrower than AAV-expression. This can be advantageous when one needs gene expression in extremely small region, such as amygdala. The other peculiar

observation using lentiviral *in vivo* expression was that it seemed to sink down much more than AAV did. When we injected lentivirus right below the pyramidal layer, the expression was also abundant in DG, which was not frequently observed when using AAV. This might be because lentivirus is much heavier than AAV, so different stereotaxic surgery coordination may have to be used for lentivirus and AAV. After modulating the coordination to above the pyramidal layer, the unwanted expression in DG reduced, though it sometimes still occurred. One will need to optimize stereotaxic coordination of oneself when using AAV and lentivirus.

In this study, we transduced LV-U6-CRISPR_GRIA2-SpCas9-P2A-tdTomato and LV-U6-CRISPR_GRIA2-SpCas9-P2A-GFP in mouse hippocampal CA1. As GluA2 deleted, AMPARs becomes GluA2 lacking AMPAR, which is calcium permeable. We confirmed that GluA2 was actually knocked out with electrophysiological studies. As we know, this is the first study of endogenous calcium permeable AMPARs of adult mice using CRISPR/Cas9 system *in vivo* expression. Furthermore, we succeeded in producing high titer LV-U6-CRISPR_GRIA2-CamKIIα-Cas9-P2A-GFP whose titer is ~$5x10^9$TU/ml. The lentivirus titer ranging between 10^9TU/ml and 10^{10}TU/ml is high enough for the CRISPR/Cas9 system to be expressed in most of the hippocampal CA1 pyramidal neurons around the injection sites. This means that behavior tests are possible after CRISPR/Cas9 system *in vivo* expression. However, it has to be tested first whether knockout phenotype by CRISPR/Cas9 is sufficient enough to affect behavioral differences.

The knockout by CRISPR/Cas9 system is advantageous over conventional experiments using transgenic mouse lines. It is possible to knockout target gene very fast by injecting the lentiviral CRISPR/Cas9 system. While it took several months to years to create conventional transgenic mouse lines, injecting lentiviral CRISPR/Cas9 system into wildtype mouse can save much time. Furthermore, the knockout phenotype can be confined to the target region of stereotaxic surgery using lentiviral CRISPR/Cas9 system, whereas knockout phenotype of many conventional knockout mouse lines generally exists in whole body or whole brain. Furthermore, it is possible to reduce unwanted effects in transgenic mouse lines, such as compensatory molecular pathways initiated by gene deletion in early developmental stage, or lethality effect, which is not difficult to observe for the GluA2 knockout transgenic mouse lines. This means that delivering CRISPR/Cas9 system via lentivirus injection can provide more physiological condition than conventional transgenic mouse lines.

In this study, we found the condition that results in high titer lentiviral CRISPR/Cas9. In case higher titer is needed, reducing the volume of DPBS for resuspension of lentiviral pellets and increasing the number of plates during transfection can be considered (Table2). This can increase lentiviral CRISPR/Cas9 titer by more than 10 times. Also, the pLenti-CRISPR v2 can be engineered to increase the virus packaging efficiency. Boris Kantor Lab recently published that they succeeded to increase lentiviral CRISPR/Cas9 titer up to 7 times, by introducing two Sp1—the transcription factor, into the pLenti CRISPR v2 (Ortinski et al., 2017;

Vijayraghavan et al., 2017). Engineering the transfer vector would be the best way to produce high titer lentiviral CRISPR/Cas9 with much less effort and material.

The lentivirus can be applied to other experiments than CRISPR/Cas9 system delivery. Lentivirus DNA cargo capacity is about 8 kb, whereas that of AAV is about 4.7kb. This makes lentivirus more appropriate gene delivery system if DNA larger than 4.7kb is required. In case of delivering genes other than CRISPR/Cas9, distinct DNA vector molar ratio during transfection may have to be considered, because every transfer vector has different optimal DNA vector molar ratio. This can be solved by referring to previous publications that produced the lentivirus one wants. As virus packaging efficiency increases as the transfer vector size decreases (Kumar et al., 2003), it is expected that titer over 10^{10}TU/ml can be produced.

There are some concerns about immune response to lentivirus. Although most of the studies indicate that lentivirus injection induces little immune response, suggesting that lentivirus is appropriate for the stable *in vivo* expression (Abordo-Adesida et al., 2005; Follenzi et al., 2007; Jakobsson et al., 2006; Nayak et al., 2010; Parr-Brownlie et al., 2015), some studies indicated that lentivirus injection in the brain increased the number of microglia in the injected sites (Kunitsyna et al., 2016). It may be due to the high titer of lentivirus as suggested by previous research (Abordo-Adesida et al., 2005). This implies that we need to be cautious of selecting the optimal titer to reduce the unwanted immune responses.

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초록

렌티바이러스를 이용한 CRISPR/Cas9의 생체 내 발현 최적화에 관한 연구

고광희

뇌과학협동과정

자연과학대학

서울대학교

Clustered Regularly Interspaced Short Palindromic Repeats /CRISPR-associated 9 (CRISPR/Cas9) 시스템은 유전체 염기 서열을 특이적으로 인식하는 능력과 유전자 편집에 있어서 유용성 때문에 과학자들에게 주목받고 있다. 신경과학에서 CRISPR/Cas9 시스템을 사용한 연구가 늘고 있음에도 불구하고, 대부분의 연구가 신경세포 일차배양이나 유기체 슬라이스 배양과 같은 시험관 내 조건에서 이루어졌다. 특이성 및 유용성 등을 고려하면 더 생리적인 체내 조건에서 이루어진 연구는 아직 많이 이루어지지 않았다.

본 연구에서는 렌티바이러스를 이용한 CRISPR/Cas9 시스템을

체내에서 발현시키는 적절한 과정을 찾아나간다. 포유류 세포 배양에 사용되는 배지, 초고속 원심 분리에 사용하는 로터의 종류, 트렌스펙션 때 사용되는 vector의 몰수 비, 그리고 transfer vector의 insert 부분의 크기 등 적정 조건을 찾아나가는 과정 동안 렌티바이러스 CRISPR/Cas9의 역가와 순도를 높임으로써 전기생리학 실험과 동물실험에 적정한 고농도의 렌티바이러스 CRISPR/Cas9을 제작하는데 성공하였다.

GluA2를 제거하기 위하여 GluA2를 인식하는 sgRNA가 사용되었고 GluA2 knockout은 웨스턴 블랏, 그리고 전기생리학 실험을 통해 확인되었다. 렌티바이러스를 통해서 CRISPR/Cas9을 생체내 발현시키는 것은 일반적인 생물학 연구에만 유용할 뿐 아니라, 의생명과학 연구에 있어서도 유용할 것으로 생각된다.

주요어: 렌티바이러스, CRISPR/Cas9 시스템, GluA1 subunit, GluA2 subunit, 칼슘 투과성 AMPAR

감사문

이 연구를 시작하게 된 계기를 마련해주시고 많은 논의를 통해 실험을 좋은 방향으로 진행하게 도와 주셨을 뿐 아니라 패치 레코딩까지 진행하신 박포정 박사님께 감사드립니다. 면역 주사를 해주신 예상현, 좋은 이미징 결과를 제공해주신 김지일 선배와 한대희, 렌티바이러스 제작 과정에서 생긴 의문점에 친절하게 답해주신 도야마 의과대학의 노리아키 오카와 박사님과 카오루 이노구치 교수님께도 감사의 말씀을 드립니다. 저의 지도교수님으로 많은 조언을 아끼지 않으신 강봉균 교수님께도 깊은 감사의 말씀 올립니다.