Note

Generation of Dopamine Transporter (DAT)-mCherry Knock-in Rats by CRISPR-Cas9 Genome Editing

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Midbrain dopaminergic neurons respond to rewards and have a crucial role in positive motivation and pleasure. Electrical stimulation of dopaminergic neurons and/or their axonal fibers and arborization has been often used to motivate animals to perform cognitive tasks. Still, the electrical stimulation is incompatible with electrophysiological recordings. In this light, optical stimulation following artificial expression of channelrhodopsin-2 (ChR2) in the cell membrane has been also used, but the expression level of ChR2 varies among researchers. Thus, we attempted to stably express ChR2 fused with a red fluorescence protein, mCherry, in dopaminergic neurons. Since dopamine transporter (DAT) gene is known as a marker for dopaminergic neurons, we inserted *ChR2-mCherry* into the downstream of the *DAT* gene locus of the rat genome by clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (CRISPR-Cas9) genome editing and created DAT-ChR2-mCherry knock-in rats. Immunohistochemistry showed that ChR2-mCherry was expressed in dopaminergic neurons in homozygote knock-in rats, whereas whole-cell recordings revealed that ChR2-mCherry-positive neurons did not fire action potentials upon blue light stimulation, indicating that ChR2 was not functional for optogenetics. Nevertheless, fluorescent labeling of dopaminergic neurons mediated by mCherry could help characterize them physiologically and histologically.

Key words dopamine transporter, mCherry, knock-in, rat, clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (CRISPR-Cas9)

INTRODUCTION

Dopamine has a pivotal role in motivational control and pleasant sensation in animals.[1)] The primary sources of dopamine in the brain are dopamine-releasing (*i.e.*, dopaminergic) neurons located in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) in the ventral midbrain. Electrical stimulation of dopaminergic neurons or their axonal fibers has been behaviorally used as a neural reward to facilitate operant and spatial learning, in which rats, not mice, are preferentially chosen because they have a potential to perform more complicated tasks.[2–5)] Nevertheless, electrical stimulation is disadvantageous when combined with electrophysiological recordings because it produces electric noises that contaminate neural signals. To avoid this issue, researchers often take a strategy of photostimulation of dopaminergic neurons after artificial expression of channelrhodopsin-2 (ChR2) in the cell membranes. Viral infection and *in utero* electroporation are often used to artificially express ChR2 in dopaminergic neurons, but the expression levels vary among animals or experimenters.

To solve this problem, we focused on genome editing techniques to stably express fluorescent protein-fused ChR2 in the dopaminergic neurons. Since dopaminergic neurons endogenously express dopamine transporter (DAT) protein, we attempted to create DAT-ChR2-mCherry knock-in rats using clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (CRISPR-Cas9) genome editing techniques.

MATERIALS AND METHODS

Ethical Approval Animal experiments were performed with the approval of the Animal Experiment Ethics Committee at The University of Tokyo (Approval Number: P29-2, P29-16, P4-10, P4-11) according to the University of Tokyo guidelines for the care and use of laboratory animals. These experimental protocols were carried out in accordance with the Fundamental Guidelines for the Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions (Ministry of Education, Culture, Sports, Science and Technology, Notice No. 71 of 2006), the Standards for Breeding and Housing of and Pain Alleviation for Experimental Animals (Ministry of the Environment, Notice No. 88 of 2006) and the Guidelines on the Method of Animal Disposal (Prime Minister's Office, Notice No. 40 of 1995). Animal experiments were also performed with the approval of the Institutional Animal Care and Use Committee of RIKEN Kobe Branch (Approval Number: A2001-03), according to the institutional guidelines for animal experimentation. All efforts were made to minimize animal suffering.

Animals and Zygotes Wistar rats and Wistar cryopre-

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served zygotes were sourced from Japan SLC and Charles River Laboratories, respectively. All animals were housed under conditions of controlled temperature and humidity (22 ± 1 °C, 55 ± 5%) and were maintained on a 12 : 12-h light/dark cycle. They were fed with a standard pellet diet and tap water *ad libitum*.

Generation of DAT-ChR2-mCherry Knock-in Rats The DAT-ChR2-mCherry knock-in rats (Accession No. CDB0100RE: https://large.riken.jp/distribution/mutant-list.html) were generated by CRISPR/Cas9-mediated knock-in in zygotes based on the mouse method as previously described[6] (Fig. 1, Supplementary Fig. 1). The gRNA site (5′-CAC TGG CTG TTG CTG TAA AG-3′) was designed by using CRISPR-direct (https://crispr.dbcls.jp),[7] and the MMEJ (microhomology-mediated end-joining)-based donor vector consisting of microhomology arms and a P2A-ChR-mCherry (plasmid #20297, addgene) was generated to insert the cassette at the 5-base upstream of the PAM sequence.

For zygote collection, 10-week-old female Wistar rats (Charles River Laboratories) were superovulated by administration of pregnant mare serum gonadotropin (PMSG) (50 IU), and 48 h later human chorionic gonadotropin (hCG) (50 IU).[8] Several hours after hCG administration, females were crossed with 12- to 48-week-old males, and one-cell stage zygotes were collected the next day. The zygotes were cultured with KSOM for rats in all procedures.[9] The mixture of two crRNAs (CRISPR RNA) (50 ng/μL), tracrRNA (*trans*-activating crRNA) (100 ng/μL), donor vector (1 ng/μL), and Cas9 protein (100 ng/μL) (A36496, Thermo Fisher Scientific, Waltham, MA, U.S.A.) was injected into the pronucleus of the zygote. DAT1-crRNA (5′-CAC UGG CUG UUG CUG UAA Agg uuu uag agc uau gcu guu uug-3′), PITCh-gRNA3-crRNA (5′-GCA UCG UAC GCG UAC GUG UUg uuu uag agc uau gcu guu uug-3′) and tracrRNA (5′-AAA CAG CAU AGC AAG UUA AAA UAA GGC UAG UCC GUU AUC AAC UUG AAA AAG UGG CAC CGA GUC GGU GCU-3′) were purchased from FASMAC (Kanagawa, Japan). For zygote transfer, female Wistar rats (10- to 16-week-old) were checked for proestrus using a vaginal impedance checker for rats (MK-12-B, Muromachi Kikai, Tokyo, Japan) the day before injection, mated with vasectomized males, and females confirmed to be plugged the next day were used for transfer of injected zygotes.

Genotyping Tissues were provided by punching/notching ears or cutting tails of F0 rats or their descendants. The tissues were lysed with proteinase K, and genomic DNA was extracted by phenol-chloroform, followed by ethanol precipitation. In another case, DNA was extracted and purified by a spin column method using the DNeasy Blood & Tissue Kit (69504, QIAGEN, Venlo, the Netherlands) as instructed in the manufacturer's protocol.

Genotyping was performed by PCR using GoTaq Green Master Mix (M7122, Promega, Madison, WI, U.S.A.) and appropriate pairs of following primers (Eurofins): 5′ gt FWD, 5′-GTG AGT GGT GCC ACC TAG TAA GTC C-3′; 5′ gt REV, 5′-GCT GAA TCC TGC TGC AAG CCA CTG C-3′; 3′ gt FWD, 5′-CCA CTA CGA CGC TGA GGT CAA GAC C-3′; 3′ gt REV, 5′-AGT AGG GAC AGG GAC ATT GTT GAC C-3′; mCherry FWD, 5′-GCT TCA AGG TGC ACA TGG AG-3′; mCherry REV, 5′-CAT GAA CTG AGG GGA CAG GA-3′ (Fig. 2, Supplementary Fig. 1B). The PCR cycling condition was as follows: 96 °C for 2 min, 35 cycles of 96 °C for 30 s, 63.5 °C for 30 s, 72 °C for 40 s. PCR products and a DNA ladder (NE-MWD50, NIPPON Genetics, Tokyo, Japan) were analyzed by electrophoresis through 2% agarose gel containing nucleic acid stain, Midori Green Advance (NE-MG04, NIPPON Genetics) and imaged using a gel documentation system (AE6914, ATTO, Tokyo, Japan).

Immunohistochemistry Homozygote knock-in rats were selected from offspring of F0 founders based on genotyping and were anesthetized with an overdose of urethane. After general anesthesia was confirmed by the lack of reflex responses to tail and toe pinch, the rats were transcardially perfused with 0.01 M phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.01 M PBS, and decapitated.[10] After decapitation, the brains were carefully removed and postfixed in 4% paraformaldehyde in PBS overnight. On the next day, the brains were coronally sectioned from the anterior region. All brains were sectioned using a vibratome at a thickness of 100 μm. All sections were blocked with 5% bovine serum albumin (BSA) and 0.3% Triton X-100 in PBS (called a blocking buffer, hereafter) for 1 h at room temperature.

Basic immunohistochemistry procedures have been de-

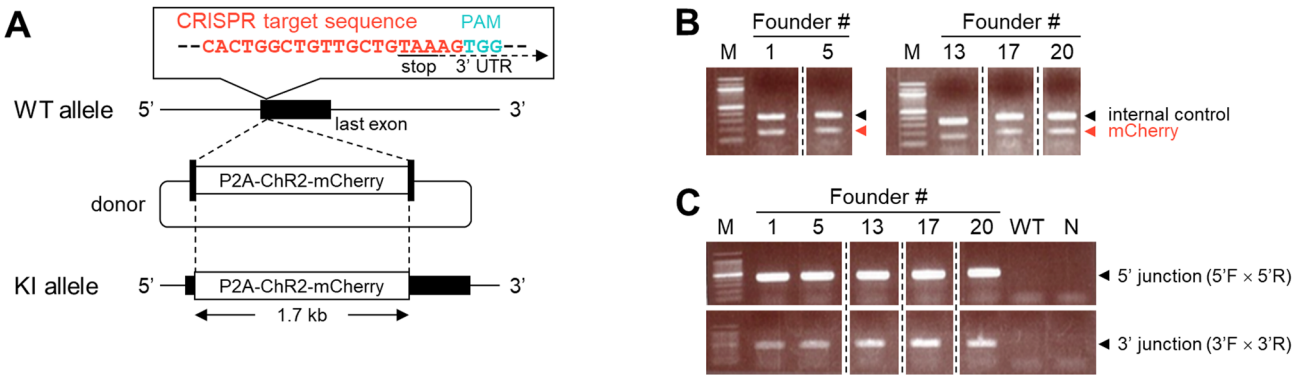

Fig. 1. Generation of DAT-mCherry Knock-in Rats

A, Schematic illustration of the genetic design of DAT-mCherry knock-in rats. B, Genotypes of five founder rats were resolved by agarose electrophoresis after PCR. PCR products encoding interleukin 2 (*black*; 324 bp) and mCherry (*red*; 161 bp) corresponded with the expected band sizes as confirmed by DNA size standards (M) used as positive controls for electrophoresis. No primer dimers were detected. C, The same as B, but for PCR products encoding 5′ junction (*top*; 390 bp) and 3′ junction (*bottom*; 443 bp). No bands were observed in the negative control (N) or wild type (WT). No primer dimers were detected. *Abbreviations*: WT, wild type; KI, knock in; PAM, protospacer adjacent motif; UTR, untranslated region; M, marker; F, forward; R, reverse; N, negative control.

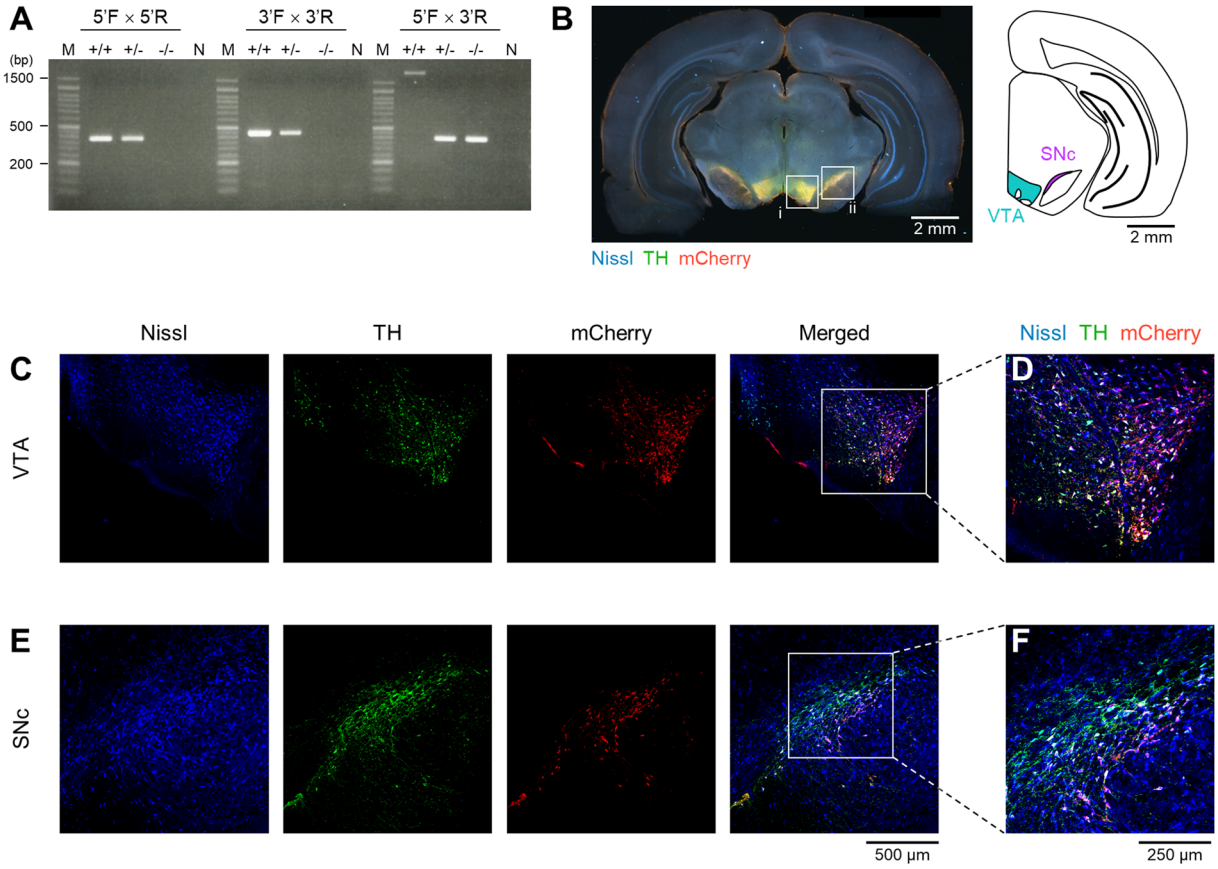

Fig. 2. Expression of mCherry in Dopaminergic Neurons in Homozygous Knock-in Rats

A, The typical electrophoresis patterns of PCR products from different genotypes: homozygous (+/+), heterozygous (+/−), and wild type (−/−) rats. Rat ear DNA was amplified with primers for 5′ junction (*left*; 390 bp), 3′ junction (*middle*; 443 bp), and the junction containing the P2A-ChR2-mCherry cassette (*right*; >1500 bp and 397 bp for knock-in and wild type alleles, respectively). These PCR products were separated on 2% agarose gels. No bands were observed in the negative controls (N) in any conditions. No primer dimers were detected. B, A representative cross-sectional view of the coronal section containing the VTA (*teal*) and SNc (*purple*) after immunostaining TH (*green*) and mCherry (*red*) and counterstaining with fluorescent Nissl (*blue*). C, Representative high-magnification images for the VTA indicated by square i in B. Images of Nissl (*first* (*leftmost*), *blue*), anti-TH (*second*, *green*), and anti-mCherry (*third*, *red*) are shown. The three signals are superimposed (*fourth*). D, A magnified image of a boxed area in *C*. The color channels are the same as C. E, The same as C, but for the SNc. F, The same as D, but for the SNc. *Abbreviations*: F, forward; R, reverse; M, marker; N, negative control; TH, tyrosine hydroxylase; VTA, ventral tegmental area; SNc, substantia nigra pars compacta.

scribed previously.[11–16] The sections were incubated with the following primary antibodies: mouse primary antibody against tyrosine hydroxylase (TH) (1 : 100; MAB318, Sigma-Aldrich, St. Louis, MO, U.S.A.) and guinea pig primary antibody against red fluorescent protein (RFP) (1 : 500; 390 004, Synaptic Systems, Göttingen, Germany) in 1% BSA and 0.3% Triton X-100 in PBS (named a dilution buffer) for approximately 16 h at 4 °C. The sections were washed with PBS for 10 min three times and then incubated with NeuroTrace 435/455 blue fluorescent Nissl stain (1 : 500, N21479, Thermo Fisher Scientific) and the following secondary antibodies: Alexa Fluor 488-conjugated goat secondary antibody against mouse immunoglobulin G (IgG) (1 : 500, A-11029, Thermo Fisher Scientific) and Alexa Fluor 594-conjugated goat secondary antibody against guinea pig IgG (1 : 500, A-11076, Thermo Fisher Scientific) in the dilution buffer for 1.5 h at room temperature.

Electrophysiology Postnatal 21- to 27-d-old knock-in rats were deeply anesthetized with isoflurane and decapitated. The brains were rapidly removed and horizontally sliced at a thickness of 400 μm using a vibratome in an ice-cold, oxygenated (95% O_2, 5% CO_2) cutting solution, which was composed of 222.1 mM sucrose, 27 mM $NaHCO_3$, 1.4 mM NaH_2PO_4, 2.5 mM KCl, 1 mM $CaCl_2$, 7 mM $MgSO_4$, and 0.5 mM ascorbic acid.[11,17] After 20 min of recovery at 35 °C, a slice was placed in a submerged chamber and was perfused at 2 mL/min at 35 °C with oxygenated artificial cerebrospinal fluid (aCSF), which was composed of 127 mM NaCl, 1.6 mM KCl, 1.24 mM KH_2PO_4, 1.3 mM $MgSO_4$, 3.0 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose. mCherry-positive neurons were visually identified under infrared differential interference contrast (IR-DIC) and fluorescence microscopy and were current-clamped using borosilicate glass pipettes (4–7 MΩ) with the K^+-based intra-pipette solution consisting of 135 mM K-gluconate, 4 mM KCl, 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), 10 mM Na_2-phosphocreatine, 4 mM Mg-ATP, 0.3 mM Na_2-GTP, 0.3 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 0.2% biocytin. The solution was adjusted to pH 7.3 and 285–290 mOsm/kg. The electrophysiological signal was amplified with a microelectrode amplifier (MultiClamp 700B, Molecular Devices) and digitized at 20 kHz by a low-noise analog-to-digital converter (Axon Digidata 1550, Molecular Devices) that worked with pCLAMP10.1 software (Molecular Devices).

At the beginning of each experiment, 800-ms rectangular currents of −200–+200 pA were injected into the cells at steps of 50 pA, and the spike responses were examined. Subsequently, using an LED illumination system (LEX2-LZ4, Brainvision), the patched neurons were exposed to blue light

to confirm whether they would be photoactivated (Supplementary Fig. 2). The parameters of the monophasic photo pulses were as follows: *photo intensity*, 10 mW; *pulse width*, 10 ms, and *interpulse interval*, 40 ms. The pulse was repeated 100 times. The series resistance of the recorded cell was determined at the beginning and end of each experiment. Data were rejected when the series resistance exceeded 35 MΩ or increased by 10%.

Following each experiment, the electrode was carefully withdrawn from the recorded neuron. The slices were perfused with 4% paraformaldehyde and post-fixed overnight. The sections were incubated with the blocking buffer (see the previous subsection for the composition) for 1 h. They were then incubated with the mouse primary antibody against TH (1 : 100; MAB318, Sigma-Aldrich) and guinea pig primary antibody against RFP (1 : 500, 390 004, Synaptic Systems) in the dilution buffer for 16 h at 4 °C, followed by incubation with the Alexa Fluor 647-conjugated goat secondary antibody against mouse IgG (1 : 500, A-21236, Thermo Fisher Scientific), Alexa Fluor 594-conjugated goat secondary antibody against guinea pig IgG (1 : 500, A-11076, Thermo Fisher Scientific), 2 μg/mL Alexa Fluor 488-conjugated streptavidin (S11223, Thermo Fisher Scientific), and 0.4% Nissl stain (N21479, Thermo Fisher Scientific) in the dilution buffer for 4 h at room temperature. The pseudocolors of TH staining and streptavidin-mediated visualization were swapped (Supplementary Fig. 2A) to be consistent with the other histological image (Figs. 2C–F); that is, green and white represent TH and a patched neuron, respectively (Supplementary Fig. 2A).

Image Acquisition and Processing All raw images were acquired using an inverted microscope (Eclipse Ti2, Nikon, Tokyo, Japan) equipped with a 4× objective lens (Plan Apo (NA, 0.2; WD, 20 mm), Nikon). To see whole coronal sections (Fig. 2B), original images (2136 × 2136 pixels) were first captured using the Eclipse Ti2 microscope equipped with a monochrome camera (DS-Qi2, Nikon). Image tiling was then automatically implemented to increase the field of view using imaging software (NIS-Element Ar, Nikon). To view specific regions of interest (Figs. 2C, E, Supplementary Fig. 2A), images (2048 × 2048 pixels) were acquired at Z-intervals of 1.0 μm using the Eclipse Ti2 microscope equipped with a confocal laser microscope system (A1 HD25, Nikon) and postprocessed using ImageJ software (National Institutes of Health).

RESULTS AND DISCUSSION

We coinjected donor plasmid vectors of P2A and ChR2-mCherry with CRISPR cocktails into pronuclei of zygotes to insert the P2A-ChR2-mCherry cassette into the DAT allele target site (Fig. 1A, Supplementary Fig. 1). From 70 injected zygotes, 26 founder rats were obtained, and PCR and sequencing revealed five of them were knock-ins (Figs. 1B, C, Supplementary Fig. 1C).

We crossed the founder and wild-type rats to produce heterozygote knock-in rats, and further crossed the heterozygote rats to obtain homozygote knock-in rats based on zygosity confirmation. We confirmed the genotypes of homozygous, heterozygous, and wild type rats based on electrophoresis patterns of PCR products (Fig. 2A). Note that the band positions of the PCR corresponded to those expected theoretically (Fig. 2A). To investigate expression of mCherry in dopaminergic neurons, we immunostained brain sections of the homozygous knock-in rats. We found coexpression of mCherry and TH in the VTA and SNc of the homozygotes (Figs. 2B–F), indicating that ChR2-mCherry was expressed in dopaminergic neurons of the homozygotes. Moreover, we observed coexpression of mCherry and TH in the perisomatic regions in the striatum, where axon terminals of dopaminergic neurons were terminated, suggesting that dopaminergic axonal fibers were also labeled by mCherry (Supplementary Fig. 2). We next electrophysiologically characterized the ChR2-mCherry-positive neurons of homozygotes using *in vitro* whole-cell recording techniques (Supplementary Fig. 3). The mCherry-positive neurons fired in response to injected currents, implicating that, at least, the firing property was preserved (Supplementary Fig. 3B). We further photostimulated the mCherry-positive neurons using blue light; however, they did not fire action potentials (Supplementary Fig. 3C).

Using CRISPR/Cas9 genome editing techniques, we created DAT-mCherry knock-in rats. We originally aimed to splice cDNA of P2A and ChR2-mCherry into rats' genes and succeeded in expression of mCherry in dopaminergic neurons. However, we failed to sufficiently express ChR2 in neurons; they were not functionally photoactivated. The failure of sufficient bicistronic (*i.e.*, ChR2 and mCherry) expression probably originates from a linker protein and/or low expression level of DAT.

We selected P2A as a linker that concatenated multiple ORFs (*i.e.*, DAT and ChR2-mCherry).[18] In contrast to IRES (internal ribosome entry site),[19] an advantage of using P2A is that the expression level of downstream ORFs is almost the same as or slightly less than that of upstream ORFs,[18] which we considered would induce enough bicistronic expression to physiologically work. However, efficiency of the self-cleavage of P2A peptides is influenced by upstream and downstream ORFs; that is, an ORF for DAT might have negatively affected the self-cleavage of P2A. Thus, if a portion of translational products of polycistron fails to be cleaved, some P2A-linked proteins can hover in the cell, not in the membrane, as a fusion protein, although ChR2 should be expressed in the cell membrane to be photoactivated. Moreover, we cannot exclude the possibility that the DAT promoter activity is not high enough to express ChR2 for optogenetics. This possibility could be partially addressed by immunohistochemistry using an antibody against ChR2,[20] although no commercially available antibody against ChR2 has been proven valid to quantitatively evaluate the expression level of ChR2 in acute brain sections.

We emphasize on the fact that mCherry fluorescence was visible in dopaminergic neurons even though ChR2-mediated optogenetics could not be employed using the rats. This fluorescent labeling of neurons is beneficial to identify dopaminergic neurons in physiological studies. As demonstrated in this study (Supplementary Fig. 3), for instance, the current knock-in rats could be used in physiological assay of dopaminergic neurons by visually targeted whole-cell recordings with the aid of fluorescence IR-DIC microscopes.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials This article contains supplementary materials.

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