DNA repair protein RAD51 enhances the CRISPR/Cas9-mediated knock-in efficiency in brain neurons.

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Abstract

Gene knock-in using the CRISPR/Cas9 system can be achieved in a specific population of neurons in the mouse brain, by using *in utero* electroporation to introduce DNA fragments into neural progenitor cells. Using this strategy, we previously knocked-in the EGFP coding sequence into the N-terminal region of the β -actin gene specifically in the pyramidal neurons in layer 2/3 of the somatosensory cortex. However, the knock-in efficiency was less than 2% of the transfected neurons. In this study, we sought to improve the knock-in efficiency using this system. First, we varied the length of the homology arms of the β -actin donor template DNA, and found that the knock-in efficiency was increased to ~14% by extending the length of the 5' and 3' homology arms to 1.6kb and 2.0kb, respectively. We then tested the effect of the DNA repair protein RAD51 and the knock-in efficiency was increased up to 2.5-fold when co-transfecting with two different β -actin and a camk2a targeting EGFP knock-in modules. The RAD51

overexpression did not alter the migration of developing neurons, density or morphology of the dendritic spines compared to those in neurons not transfected with RAD51. RAD51 expression will be useful for increasing the knock-in efficiency in neurons *in vivo* by CRISPR/Cas9-mediated homology directed repair (HDR).

Keywords: CRISPR/Cas9, homology directed repair, RAD51, *in utero* electroporation, β -actin, *camk2a*

Introduction

In utero electroporation is a powerful technique that enables genes to be introduced into neurons in specific cortical layers, by selecting the timing of electroporation into the neural progenitor cells residing in the ventricular zone [1-3]. This technique has been used to visualize the structures of neurons and to examine the function of genes in specific cortical layers, by introducing plasmid constructs expressing fluorescent marker proteins, wild-type or mutant proteins, or RNAi. Despite the many advantages of this technique, however, the possibility of artificial effects cannot be ruled out, because it involves the introduction of multiple copies of gene-expression constructs.

To overcome this problem, we previously developed a technique for knocking-in genes using the homology directed repair (HDR) mechanism of CRISPR/Cas9-mediated genome editing, combined with *in utero* electroporation [4]. We successfully applied this strategy to knock-in EGFP in-frame into the N-terminal region of the β -actin gene, specifically in a small population of pyramidal neurons in layer 2/3 of the somatosensory cortex in mice. The endogenous β -actin distribution pattern revealed strong EGFP signals in the dendritic spines of these neurons. Moreover, the density and electrophysiological properties were unaltered in the neurons overexpressing the EGFP-tagged β -actin expression construct. Other laboratories have performed similar studies [5, 6]. However, the limitation of this technique is that the knock-in efficiency is low, which restricts its application. For example, extensive single-cell genotyping will be required to look for a few knock-in neurons from all transfected cells when introducing a point mutation of gene which does not have visible knock-in markers.

Several factors have been shown to influence the knock-in efficiency by CRISPR/Cas9-mediated genome editing, including the expression levels of Cas9 protein and the sgRNA(s), the amount of repair template, the homology arm length, and the activation or inactivation of molecular machineries for DNA repair [6-8]. The DNA repair protein RAD51 is known to play a central role in HDR. After double-strand breaks (DSBs) are introduced by the Cas9 protein, RAD51 is recruited to the single-strand DNA (ssDNA) created at the end of the break point and coats the ssDNA as a multimer to form a nucleoprotein filament. This RAD51 filament binds to the homology sequence of the donor DNA, and enables homologous recombination to proceed. A chemical called RS-1 that stimulates RAD51 was shown to enhance the knock-in efficiency by HDR in cultured cells [8, 9]. Overexpressing RAD51 was also shown to increase the knock-in efficiency by HDR in human embryonic stem cells and induced pluripotent stem cells [10].

In this study, we sought to increase the knock-in efficiency attained by the CRISPR/Cas9 system and *in utero* electroporation in brain neurons. We modified the conditions of our previous procedure, which introduced EGFP into the N-terminal site of the β -actin coding region with efficiency under 2%. Here, by extending the homology arms of the donor template, we obtained a knock-in efficiency of ~14% of the transfected neurons. When co-transfecting with RAD51, the knock-in efficiency was increased up to 25% (> 10 times the efficiency using the original conditions). We also show the RAD51 increase EGFP knock-in efficiency up to 2.5-fold with different sgRNA and donor vector constructs for β -actin and camk2a. This improvement will broaden the application of this technique in the neuroscience field.

Materials and Methods

Construction of plasmid vectors

A 1753-bp fragment carrying the EGFP cDNA, and the 508-bp upstream and 530-bp downstream genomic sequences from the translation initiation codon of β -actin was amplified by PCR with primers pair A using pBSSK-EGFP- β -actin-donor (0.5kb-0.5kb) [4] as a template. The 471-bp genomic fragment was amplified by PCR with primers pair B using ICR mouse

genomic DNA as a template. These two DNA fragments were cloned into the XhoI-NotI site of pBluescript II SK(+) (Stratagene) using the GeneArt Seamless Cloning and Assembly Kit (Invitrogen) to yield 0.5kb-1.0kb donor. The 1641-bp genomic fragment was amplified by PCR with primers pair C using ICR mouse genomic DNA as a template and cloned into the BglII site of pBSSK-EGFP- β -actin-donor to yield 0.5kb-2.0kb donor. The 1168-bp genomic fragment was amplified by PCR with primers pair D, and cloned into the SacI site of pBSSK-EGFP- β -actin-donor to yield 0.5kb-1.6kb donor. The 1641-bp genomic fragment was amplified as described above and cloned into the BglII site of 0.5kb-2.0kb donor to yield 1.6kb-2.0kb donor. The entire coding sequence of TagRFP was amplified by PCR using pEBMulti-TagRFP [4] as a template, and cloned into pEBMulti-Neo (Wako) to yield pEBMulti-TagRFP. pCGSapI- β -actin-sgRNA#2 and pCGSapI-LacZ-sgRNA that contain Cas9 expression cassette and sgRNA for β -actin or lacZ were described previously [4]. pAAV-HDR-mEGFP-Actin, pAAV-HDR-mEGFP-camk2a, and pAAV-EFS-SpCas9 were obtained from addgene (#119870, #104589, and #104588, respectively) [11]. All the primers' sequences are listed in Supplementary Table.

***In utero* electroporation.**

In utero electroporation was performed essentially as described previously [4, 12]. Briefly, pregnant ICR mice at embryonic day 15.5 (E15.5) were anesthetized with ketamine (100 mg/Kg, i.p.) and xylazine (10 mg/Kg, i.p.). Approximately 1 μ l of DNA solutions containing 0.01% fast green were injected into the lateral ventricles of embryos. The DNA solutions contained 1.5 μ g/ μ l donor plasmid, 1.0 μ g/ μ l pEBMulti-TagRFP, and 1.5 μ g/ μ l pCGSapI- β -actin-sgRNA#2 [4] or pCGSapI-LacZ-gRNA [4]. For RAD51 co-expression, 2 μ g/ μ l expression vector for mouse RAD51 (pCAG-RAD51) (gifted by Dr. M. Abe) was added to the DNA solution. DNA solution containing 1.0 μ g/ μ l pEBMulti-TagRFP, 1.5 μ g/ μ l pAAV-EFS-SpCas9, 1.5 μ g/ μ l pAAV-HDR-mEGFP-Actin or 1.5 μ g/ μ l pAAV-HDR-mEGFP-camk2a with or without 2 μ g/ μ l pCAG-RAD51 were also injected for testing additional sites of β -actin and camk2a. Each injected embryo was subjected to six square electric pulses (43 V, 50 msec, 1 Hz) using a tweezers-type electrodes (CUY650P5; NEPA Gene) connected to an electroporator (CUY21E; NEPA Gene). Transfected pups were identified at postnatal days 0-2 (P0-2) by TagRFP signals through the scalp. The animal

protocol was approved by the Animal Care and Use Committee of Shinshu University. The methods were carried out in accordance with the Regulations for Animal Experimentation of Shinshu University.

Genomic PCR and DNA sequencing

Brains were dissected from in utero electroporated and non-electroporate control mice. Transfected areas expressing TagRFP signals were excised by razor blade and the brain tissues were incubated in the proteinase K solution (1:100, Sigma-Aldrich) at 50 °C for overnight. RNase A (200, Nacalai) were added and incubated at 37 °C for 1 hour. Genomic DNAs were extracted and purified by phenol/chloroform extraction followed by ethanol precipitation. Genomic PCR were performed using primer sets a-b (for detecting 5' donor-genome junction for pAAV-HDR-mEGFP-Actin), c-d (for detecting 3' donor-genome junction for pAAV-HDR-mEGFP-Actin), e-b (for detecting 5' donor-genome junction for pAAV-HDR-mEGFP-camk2a), and c-f (for detecting 3' donor-genome junction for pAAV-HDR-mEGFP-camk2a) with a step-down PCR, whose annealing temperature decreasing from 74 °C to 68 °C. PCR products were examined by electrophoresis on 0.7% agarose gel and DNA sequencing with BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). All primer sequences are listed in the Supplementary Table.

Histological analysis.

Under deep anesthesia, P14-16 mice were perfused and fixed with 4% paraformaldehyde in phosphate-buffered saline, pH 7.4. The fixed brain was cut to 100-µm-thick coronal sections with a micro slicer (VT1200S; Leica Biosystems). Sections were stained with DAPI and mounted. Fluorescence images were acquired with a confocal laser-scanning microscope (TCS SP8; Leica Microsystems)

Statistical analysis.

Statistical significance was evaluated by Student's t-test (for two groups) or one-way ANOVA followed by Bonferroni's post-hoc test (for multiple groups). Statistical significance was assumed when $p < 0.05$.

Results

CRISPR/Cas9-mediated EGFP knock-in at the β -actin locus in cortical layer 2/3 pyramidal neurons with homology arms of various lengths

In our previous study, we used a donor vector containing 0.5kb-0.5kb homology arms on both sides flanking the EGFP coding sequence. Tsunekawa et al. showed that extending the homology arms of the EGFP donor vector from 100bp to 1.8kb significantly increased the efficiency of its knock-in into the *blt1* tubulin gene by *in utero* electroporation of mouse embryos at E15.5 [6]. To examine whether longer homology arms would increase the HDR-mediated knock-in efficiency at the β -actin locus, we constructed donor plasmids carrying EGFP cDNA flanked by homology arms of various lengths, to knock-in the EGFP cDNA immediately after the first ATG codon of the β -actin gene and generate an EGFP- β -actin fusion protein (Fig. 1A). pEBMulti-TagRFP, pCGSapI- β -actin-sgRNA, and donor plasmids with homology arms of various lengths were introduced into neural progenitor cells in the ventricular zone at E15.5 by *in utero* electroporation (Fig. 1B). We electroporated 8~10 embryos per litter and more than 80% of electroporated pups were recovered judged by the expression of RFP signals in most of our experiments. Our previous study showed that the knock-in of EGFP cDNA at the β -actin gene locus resulted in the expression of an EGFP- β -actin fusion protein [4]. To evaluate the knock-in efficiency of EGFP cDNA at the β -actin gene locus, we determined the percentage of EGFP-positive neurons in total TagRFP-positive neurons. We first compared the percentage of EGFP-positive neurons in mice transfected with 3' arms of 0.5, 1.0, and 2.0kb (Fig. 1A). Under all the conditions, TagRFP fluorescence signals were observed in pyramidal neurons in layer 2/3 of the somatosensory cortex at P14-P16. In contrast, EGFP fluorescence signals were detected only in a sparse population of the cortical layer 2/3 pyramidal neurons in the mice transfected with pCGSapI- β -actin-sgRNA and donor plasmids (Fig. 2A). The percentage of transfected neurons that were EGFP-positive increased when the donor template DNA had longer 3' arms (Fig. 2A-B; $2.51 \pm 0.38\%$ with 0.5kb-0.5kb, $3.20 \pm 0.67\%$ with 0.5kb-1.0kb, and $9.64 \pm 1.04\%$ with 0.5kb-2.0kb). Next, we examined the effect of the 5' arm length on the knock-in efficiency of EGFP cDNA. Approximately 14% of the transfected neurons were EGFP-positive when the donor plasmid carried 1.6kb of 5' and 2.0kb of 3' arms (Fig. 2A, B; $13.73 \pm 0.72\%$). This percentage of EGFP-positive neurons was higher than that obtained with other donor plasmids (5.47 times that obtained with 0.5kb-0.5kb, $P = 0.00014$; 4.29 times that with 0.5kb-1.0kb, $P = 0.00043$;

and 1.42 times that with 0.5kb-2.0kb, $P = 0.03148$; Bonferroni's post hoc test) (Fig. 2A, B). No EGFP fluorescence signals were detected in the cortical layer 2/3 pyramidal neurons in mice transfected with control LacZ-targeting sgRNA (Fig. 2A, B). These results indicated that increasing the length of the 5' and 3' homology arms of the HDR-mediated donor greatly enhanced the knock-in efficiency.

RAD51 overexpression in neural progenitor cells enhances the CRISPR/Cas9-mediated EGFP knock-in efficiency at the β -actin locus in cortical layer 2/3 pyramidal neurons

RAD51 overexpression is reported to enhance HDR in vitro [10]. To examine whether RAD51 also enhances HDR in vivo, we co-transfected pCAG-RAD51, an expression vector for mouse RAD51, with the pEBMulti-TagRFP, pCGSapI- β -actin-sgRNA, and pBSSK-EGFP- β -actin-donor-1.6kb-2.0kb into cortical progenitor cells in the ventricular zone at E15.5 by *in utero* electroporation. The percentage of EGFP-positive neurons in total TagRFP-positive neurons was 1.76-fold increased in the RAD51-overexpressing condition compared to the condition without RAD51 overexpression (Fig. 3A, B) ($13.73 \pm 0.76\%$ in the case without RAD51, $24.20 \pm 3.18\%$ in the case with RAD51, $P = 0.03555$, Student's t test). The CRISPR/Cas9-mediated EGFP knock-in efficiency at the β -actin locus was enhanced approximately 10-fold by the RAD51 overexpression and longer homology arms compared to the original condition in which a donor vector with 0.5kb-0.5kb homology arms was used (Fig. 2B and Fig. 3B). The laminar distribution (Fig 3C) and the spine density (Fig 3D and E) of the RAD51-overexpressing neurons were unaltered compared to those without RAD51 overexpression, suggesting that the RAD51 did not affect neuronal development.

RAD51 also enhances the EGFP knock-in efficiency with other CRISPR/Cas9 and donor vectors.

To examine whether the RAD51 effect is common for other CRISPR/Cas9-mediated HDR, we overexpressed RAD51 with pAAV-HDR-mEGFP-Actin; another sgRNA and EGFP donor construct for β -actin gene, or pAAV-HDR-mEGFP-camk2a; sgRNA and the EGFP donor for camk2a gene [11] (Fig. 4A). Like above results, RAD51 increases 2-fold in the knock-in with pAAV-HDR-mEGFP-Actin (Fig. 4B, C) and 2.5-fold in the knock-in

with pAAV-HDR-mEGFP-camk2a (Fig. 4D, E) compared to the control without RAD51 overexpression. We confirmed that EGFP were knocked-in to the proper region by HDR, not NHEJ, by genomic PCR using outside and inside primers designed in host genomic and donor vector regions (Fig.4A) followed by DNA sequencing of the PCR products (Fig.4F, G).

Discussion

To improve the knock-in efficiency in mouse neurons using *in utero* electroporation and the CRISPR/Cas9 system, we tested the effects of altering the size of the homology arms of the donor vector and overexpressing the DNA repair protein RAD51. Extending the homology arms increased the knock-in efficiency to ~14%, and overexpressing RAD51 with the extended homology arms increased it to ~25% of the transfected neurons. The knock-in efficiency varies depending on the genes involved. Mikuni et al. also targeted the N-terminal region of β -actin but knocked-in the HA epitope tag [5]. They obtained a knock-in efficiency of 3.4% by electroporation at E12. Introducing shorter fragments and performing electroporation at earlier stages generally result in higher knock-in efficiencies. The relatively low knock-in efficiency found by Mikuni et al. may have been due to their use of short single-strand oligo DNA (ssODN) as a donor fragment. Although ssODN is proven to elicit a comparative or even better homologous recombination efficiency to double strand DNA (dsDNA), a major limitation of ssODN is that the maximum length can only be ~200 bp. Thus, having a donor fragment with longer homology arms may be more effective than using ssODN. The longest homology arms we tested were a combination of 1.6kb and 2.0kb. It is possible that the knock-in efficiency would be further increased using even longer arms than these. However, the copy number of the transfected donor vector could decrease due to the larger size of the plasmid. In this regard, Tsunekawa et al. reported that, in their system, the knock-in efficiency did not significantly increase when the arm length became longer than 1.0kb [6].

HDR occurs as an alternative pathway to non-homologous end joining (NHEJ) after DSBs. The HDR process is explained by analogy to the steps of synapse formation, as follows: 1) presynapse formation at the terminals of DSBs, 2) formation of a synaptic complex between the presynaptic terminal and donor

fragment, and 3) recombination. RAD51 coils around the ssDNA created at the 3' end of a DSB by the MRN complex to form a nucleoprotein filament (presynaptic filament). This presynaptic filament searches for the homologous sequence and binds to a single-strand DNA by forming a D-loop structure at the donor site (synaptic complex formation) followed by recombination. RAD51 functions as a central molecule through all these steps, supported by BRCA2, RAD52, and RAD51 paralogue complexes [9, 13-17]. Our results indicate that overexpressing RAD51 promotes these processes, to enhance the knock-in efficiency during neurogenesis. NHEJ occurs with much higher probability than HDR [18-20]. Protecting DNA fragments from exonucleolytic degradation by binding to ssDNA may also help increasing knock-in efficiency. NHEJ is coordinated by several proteins such as the Ku protein complex Ku70/Ku80, DNA-PKcs, XRCC4, and DNA ligase IV [21]. Inhibiting the NHEJ pathway is reported to increase the efficiency of HDR [10, 22, 23]. It would be intriguing to test the effects of activating HDR or inactivating NHEJ on CRISPR/Cas9-mediated knock-in efficiency, by overexpressing or knocking down molecules involved in these processes, respectively.

HDR is thought to occur at the S-G2 phase of the cell cycle. However, Nishimura et al. recently demonstrated that CRISPR/Cas9-mediated knock-in can also occur in adult mouse brain when the components are introduced by adeno-associated virus (AAV) [11]. If RAD51 is also effective in adult brain, it would broaden the application of this technique from basic neuroscience research to the treatment of human genetic diseases. We did not detect any morphological abnormalities in the migration and dendritic spine in RAD51-overexpressing neurons, but more careful validation to rule out toxicity will be required for clinical applications.

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Author contributions

TU, DNB, TY, and KT designed the experiments. TK and MB performed the *in utero* electroporation. TK constructed the plasmid vectors. TK, MS, EKS, TH, TM, and XL performed the histological analyses. TY, DNB, TM, and KT wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Figure legends

Figure 1. Experimental strategy for EGFP knock-in into the β -actin locus using CRISPR/Cas9-mediated HDR and *in utero* electroporation.

A, Genomic organization of mouse β -actin gene and EGFP knock-in donor vectors are shown. sgRNA targeting near initial coding region located in exon 2 of β -actin gene was used to induce DSBs by Cas9 protein. The sizes of 5' and 3' arms of donor plasmids (0.5kb-0.5kb, 0.5kb-1.0kb, 0.5kb-2kb, and 1.6kb-2kb) are shown on the left of each donor vector. B, Time course and DNA constructs for gene knock-ins are shown. In utero electroporation was performed at E15.5 to introduce DNA constructs into the pyramidal neurons of layer 2/3 of the somatosensory cortex of mouse brains. The knock-in efficiency was analyzed at P14-16 of transfected mice.

Figure 2. Extending the homology arms of the donor DNA fragment increases the knock-in efficiency of EGFP into the β -actin site.

A, Representative images of the somatosensory cortex from mice transfected with EGFP donor constructs with different homology arm lengths. Coronal brain sections counterstained with DAPI. sgRNA for LacZ was used instead of β -actin as a negative control in the bottom panel. Scale bar represents 100 μ m. B,

Summary graph of the knock-in efficiency using different donor vectors and control. The percentage of TagRFP-positive cells that were EGFP-positive (three mice per each condition were tested) is shown. The knock-in efficiency using a donor vector with 1.6kb and 2.0kb homology arms reached ~14%. No EGFP positive cells were detected when sgRNA for LacZ was used (N.D.). All values represent the mean $\pm$ SEM. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$; one-way ANOVA followed by Bonferroni's post-hoc test.

Figure 3. Co-transfection of RAD51 increases the knock-in efficiency of EGFP into the β -actin site.

A, Representative images of the somatosensory cortex from mice transfected with a 1.6kb-2.0kb donor vector with or without a RAD51 expression construct. Coronal brain sections counterstained with DAPI. Scale bar represents 100 μ m. B, Summary graph of the knock-in efficiency with or without the RAD51 expression construct. The percentage of TagRFP-positive cells that was EGFP-positive (three mice per each condition) is shown. The knock-in efficiency with the RAD51 expression vector reached ~25%. No EGFP positive cells was detected when sgRNA for LacZ was used (N.D.). C, Summary graph of laminar distribution of EGFP positive cells. Laminar distribution of EGFP knocked-in cells was unchanged with RAD51 overexpression. Six mice for RAD51 transfected and 5 mice for RAD51 untransfected were analyzed. D and E, Representative images (D) and summary graph (E) of dendritic spines of EGFP knock-in neurons with (3 mice) or without (4 mice) RAD51 co-transfection. Spine density was unchanged with RAD51 overexpression. Scale bar represents 5 μ m. N.D.; not detectable. All values represent the mean $\pm$ SEM. ** $p < 0.01$, n.s., not significant; Student's t-test was performed for detecting statistical significance.

Figure 4. Co-transfection of RAD51 increases the knock-in efficiency in other sgRNA and donor constructs.

A, pAAV-HDR-mEGFP knock-in donor vectors and the target genomic regions for β -actin and camk2a are shown. Arrows with small cases (a – f) indicate PCR primers to confirm HDR. B, Representative images of the somatosensory cortex with pAAV- β -actin-sgRNA-EGFP donor counterstained with DAPI (blue). or pAAV-camk2a-sgRNA-EGFP donor. Each transfection was performed with or without RAD51. C, Summary graph of the EGFP knock-in efficiency in β -actin gene. RAD51 increases knock-in efficiency ~ 2-fold. Five and three mice were

used with or without RAD51 overexpression, respectively. D, Representative images of the somatosensory cortex with pAAV-camk2a-sgRNA-EGFP donor. Each transfection was performed with or without RAD51. E, Summary graph of the EGFP knock-in efficiency in camk2a gene. RAD51 increases knock-in efficiency ~ 2.5-fold. Seven and eight mice were used with or without RAD51 overexpression, respectively. F and G, Genomic PCR and sequencing results for confirming HDR in β -actin EGFP knock-in (F) and camk2a EGFP knock-in (G). Indicated primers in Fig. 4A were used to amplify the junction regions of β -actin, e and f for camk2a. DNA fragments (left) and representative sequence chromatograms around the junction regions between host genome and donor plasmids are shown (right). – and + on the agarose gel bands indicate without and with RAD51 co-transfection, respectively. NC indicates negative control from wild-type mouse sample without in utero electroporation. (–) and (+) on the left of sequencing results indicates without and with RAD51 co-transfection, respectively. These results indicate that EGFP are successfully knocked-in both β -actin and camk2a loci by HDR.

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A **B**

Supplementary Table Primers used in this study

Primer name	Sequence	Ref
Primers for the construction of plasmid vectors		
Pair A Forward (Fwd)	GACTCACTATAGGGCTGGGATGCCACTGGCGGTGC	this study
Pair A Reverse (Rev)	GACAATTGAGAAAGGCGGTGG	this study
Pair B Fwd	CCACGCCCTTTCTCAATTGTC	this study
Pair B Rev	ACCCTCACTAAAGGGGACAGCACAGCCTGGATGGC	this study
Pair C Fwd	CATTGTTACCAACTGGGACG	this study
Pair C Rev	ACCCTCACTAAAGGGTCTGCGCAAGTTAGGTTTGTG	this study
Pair D Fwd	GACTCACTATAGGGCGGCTTTCGGGCTATTGCTAG	this study
Pair D Rev	GCGCAGCTAACTAGGAAGAG	this study
Primers for genomic PCR		
β -actin PCR Fwd (a)	CTGGCTTTCGGCTATTGCT	this study
GFP PCR Rev (b)	GCTGAACTTGTTGGCCGTTTAC	ref.11
GFP PCR Fwd (c)	CATGGTCCTGCTGGAGTTCGTG	ref.11
β -actin PCR Rev (d)	GGGAGAGCATAGCCCTCGTA	ref.11
camk2a PCR Fwd (e)	AGCACAGCAGAAAAC TG GGA	ref.11
camk2a PCR Rev (f)	GCACCAGTGTTTGCCCTTTC	this study
Primers for Sanger sequencing		
β -actin sequence Fwd	GCTGGTGATAAGTGGCCTTG	this study
β -actin sequence Rev	GCTAGGCGTAAAGTTGGCTG	this study
camk2a sequence Fwd	CAGAAAGAGCAGCAGGCTACA	this study
camk2a sequence Rev	TCCTCTGGGAATTGGTCTG	this study