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Original Article

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Neuronal Expression and Cell-Type-Specific Gene-Silencing of Best1 in Thalamic Reticular Nucleus Neurons Using pSico-Red System

Jae-Young Jung[1,3], Seung Eun Lee[2], Eun Mi Hwang[1,3] and C. Justin Lee[1,3,4]*

[1]Center for Neuroscience and Functional Connectomics, Brain Science Institute, Korea Institute of Science and Technology (KIST), Seoul 02792, [2]Research Animal Resource Center, Korea Institute of Science and Technology (KIST), Seoul 02792, [3]Neuroscience Program, University of Science and Technology (UST), Daejeon 34113, [4]KU-KIST School of Converging Science and Technology, Korea University, Seoul 02841, Korea

Assessing the cell-type expression pattern of a certain gene can be achieved by using cell-type-specific gene manipulation. Recently, cre-recombinase-dependent gene-silencing tool, pSico has become popular in neuroscientific research. However, pSico has a critical limitation that gene-silenced cell cannot be identified by fluorescence, due to an excision of the reporter gene for green fluorescence protein (GFP). To overcome this limitation, we newly developed pSico-Red, with *mCherry* gene as a reporter outside two loxP sites, so that red mCherry signal is detected in all transfected cells. When a cell expresses cre, *GFP* is excised and shRNA is enabled, resulting in disappearance of GFP. This feature of pSico-Red provides not only cell-type-specific gene-silencing but also identification of cre expressing cells. Using this system, we demonstrated for the first time the neuronal expression of the Bestrophin-1 (Best1) in thalamic reticular nucleus (TRN) and TRN-neuron-specific gene-silencing of Best1. We combined adeno-associated virus (AAV) carrying Best1-shRNA in pSico-Red vector and transgenic mouse expressing cre under the promoter of distal-less homeobox 5/6 (DLX5/6), a marker for inhibitory neurons. Firstly, we found that almost all of inhibitory neurons in TRN express Best1 by immunohistochemistry. Using pSico-Red virus, we found that 80% of infected TRN neurons were DLX5/6-cre positive but parvalbumin negative. Finally, we found that Best1 in DLX5/6-cre positive neurons were significantly reduced by Best1-shRNA. Our study demonstrates that TRN neurons strongly express Best1 and that pSico-Red is a valuable tool for cell-type-specific gene manipulation and identification of specific cell population.

Key words: bestrophin-1, thalamic reticular nucleus, pSico, virus, DLX-cre, parvalbumin

INTRODUCTION

Bestrophin1 (Best1) is one of the Ca^{2+}-activated anion channels [1], which was firstly identified from the human eye disease, Best vitelliform macular dystrophy [2]. Best1 was shown to be widely expressed in the mouse hippocampus and cerebellum, with specific expression in astrocytes [3-6]. Best1 exhibits high

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*To whom correspondence should be addressed.
TEL: 82-2-958-6940, FAX: 82-2-958-6919
e-mail: cjl@kist.re.kr

permeability to several large ions and osmolytes such as GABA and glutamate [4, 5, 7, 8]. Especially, a series of recent studies revealed that astrocytic Best1 is responsible for tonic inhibition by tonically releasing GABA, because basal Ca^{2+} level in an astrocyte is enough to activate this channel [3, 4]. However, whether Best1 is expressed in neurons in the brain and neuronal Best1 can also be a pathway for tonic release of GABA have not been investigated yet.

Among many brain regions, thalamic reticular nucleus (TRN) is known to be composed of exclusively GABAergic interneurons [9]. Thus, TRN neurons exert a potent inhibitory action through synaptically releasing GABA at their axon terminals [10-12]. On the other hand, tonic inhibition current has not been observed in TRN neurons [13]. This is consistent with the fact that TRN neurons do not express the subunits that mediate tonic inhibition current [14]. In contrast, TRN neurons receive synaptic input from cerebral cortex, in which cortical neurons possess δ subunit-containing extra-synaptic $GABA_A$ receptors, which are critical for generating $GABA_A$ receptor-mediated tonic inhibition current [14, 15]. According to a recent report, extracellular GABA in dentate gyrus can inhibit perforant pathway neurons projecting to this area via presynaptic $GABA_A$ receptor located in the axon terminals of the perforant fibers [16]. Therefore, $GABA_A$ receptors containing δ subunit in the axon terminals of the cortical neurons projecting to TRN might sense tonically released GABA in the TRN. Therefore, we raise a possibility that tonic GABA release might exist in TRN neurons and that it can be released through neuronal Best1.

In order to study the role of neuronal Best1 for tonic inhibition in TRN region, which contains heterogeneous interneuronal populations, we opted for gene-silencing technique for manipulating *Best1* mRNA expression in a specific cell-type. Cre-loxP recombination system is usually used for cell-type-specific gene-silencing studies [17]. Small hairpin-forming RNA interference is commonly used for knockdown of certain gene expression [18]. Recently, Ventura and his colleagues developed pSico vector for stable expression of shRNA, with conditional excision of shRNA cassette by cre recombinase [19]. This system has been widely utilized for cell-type-specific gene-silencing [19-22]. However, pSico has a critical limitation that gene-silenced cells cannot be identified by fluorescence, due to the excision of the reporter gene. To circumvent this critical issue, we developed an advanced version of pSico, we named, pSico-Red. In pSico-Red vector we added a red colored mCherry, which is always present in the transfected cells. In this way, mCherry will be expressed in every transfected cells, whereas GFP will be expressed depending on the presence of cre. In this study, we have developed pSico-Red and characterized it for the study of Best1 expression in TRN neurons.

MATERIALS AND METHODS

Animals and housing

Mice were allowed free access to food and water, and were maintained under a 12-h light/dark cycle, with the light cycle beginning at 8:00 AM. Animals were cared for and handled in accordance with institutional guidelines of the Korea Institute of Science and Technology, Seoul, Korea. DLX5/6-cre [23] mice and C57BL6/J mice were used.

Construction of pSico-Red: pAAV-Sico-Red Adeno-Associated Virus (AAV) vector

The general construction strategy for pSico-Red is described in Fig. 1a. For cre-dependent gene-silencing, U6-loxP-CMV-GFP-loxP-shRNA sequence from pSico lentiviral vector (addgene, UK) was amplified by PCR with MluI or BstB1-AflII-BglII-containing specific primers, and then it was cloned into pAAV-MCS vector (Stratagene, USA). To overcome disadvantage of pSico vector, cre-dependent removal of CMV-GFP sequence, additional EF1a promoter and mCherry sequences were sequentially added into MluI site of first cloned pAAV vector (pAAV-Sico) through the MluI, BstB1 and AflII restriction sites.

Best1 shRNA and scrambled shRNA, and virus production

The Best1 nucleotides from 774 to 793(5'-tttgccaacttgtcaatgaa-3') was selected for target region of Best1 shRNA as previous report [5]. For adeno-associated-virus (AAV)-based shRNA expression, Best1 shRNA was synthesized as follows: 5'-tttgccaacttgtcaatgaat tcaagagatcattgacaagttggcaatttttc-3' and 5'-tcgagaaaaaatcgcatagcg tatgccgtttctcttgaaaacggcatacgctatgcgaa-3'. The annealed double-stranded oligo was inserted into HpaI-XhoI restriction enzyme sites of pAAV-Sico-Red vector and verified by sequencing. Scrambled shRNA-containing pAAV-Sico-Red construct was used as control. Using these viral vectors, AAV was packaged at KIST Virus Facility (http://virus.kist.re.kr).

Stereotactic surgery procedure

Mice were anesthetized using 2% avertin. Mice were placed in a stereotaxic (Kopf instrument, USA) and the skull was exposed surgically. Bilateral craniotomies were performed using 0.5 mm diameter drill (SAESHIN, Korea) and the viruses were injected using a 33 gauge blunt needle (WPI, USA) or a glass micropipette attached to a 10 μl Hamilton micro syringe (Hamilton, Switzerland) filled with distilled water. A micro syringe pump (KD Scientific, USA) and its controller were used to maintain the

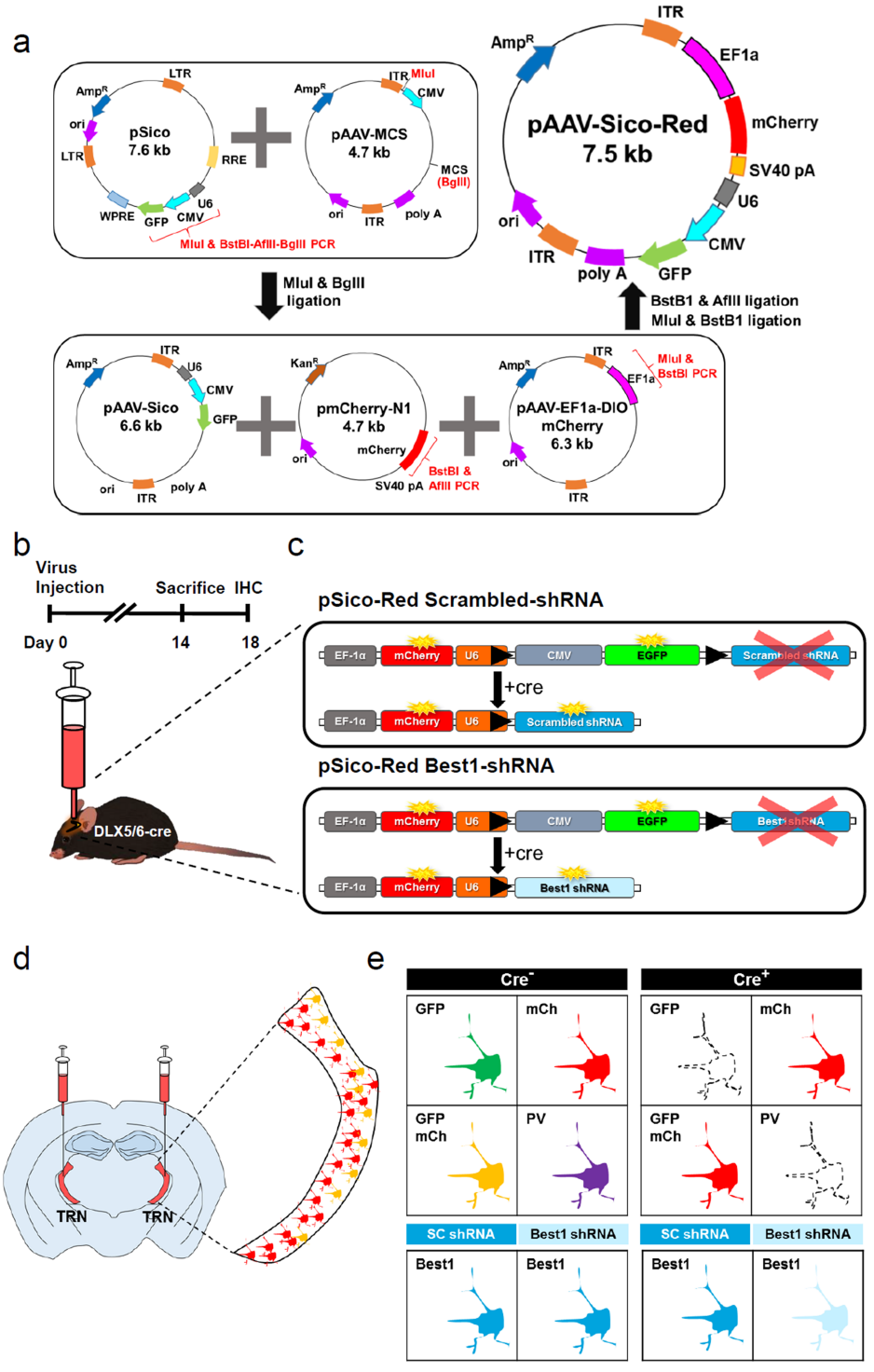

Fig. 1. Construction of pSico-Red and experimental plan. (a) Construction of pAAV-Sico-Red vector. (b) Experimental timeline and virus injection into TRN of DLX5/6-cre mouse. (c) Black diagrams for each vector containing Scrambled-shRNA or Best1-shRNA. Yellow lightening label indicates gene expression and red X-label indicates that gene is not expressed. (d) Schematic diagram of bilateral injection and distribution of cell types. Red cells represent only mCherry$^+$ cells and yellow cells represent both GFP$^+$ and mCehrry$^+$ cells. (e) Schematic representation of imaging pattern for two types of cells (Cre$^-$ and Cre$^+$). GFP and mCherry signals come from virus reporter genes. PV and Best1 expression patterns come from immunostaining. Dotted lines indicate that gene or protein is not expressed.

injection speed of 0.25 μl/min. The needle was slowly lowered to the TRN (coordinate A.P.: 1.8, M.L.: 2.2, D.V.:3.7) and remained for 4 min after moving up and down for 3 times. Then, 2 μl of viruses (diluted by 1:3 ratio) are injected to the target site. After injection, the needle stayed for additional 4 minutes, before it was slowly withdrawn. Finally, mice's scalp was closed by 9 mm auto clip (MikRon Clay Adams, USA). Two weeks after virus injection, mice were sacrificed and brains were processed for immunohistochemistry (Fig. 1b).

Immunohistochemistry

Mice were euthanized by overdosing with 2% avertin and

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perfused transcardially with 30 ml of phosphate buffered saline (PBS), followed by 30 ml of 4% paraformaldehyde (Sigma-Aldrich, USA) dissolved in PBS. Brains were extracted from the skulls and incubated in 4% paraformaldehyde at 4°C overnight. Brains were transferred to 30% sucrose solution. When brain sank to the bottom, brains were covered with optical cutting temperature (Scigen, Singapore) compound and frozen. Frozen brains were sliced at 30 μm coronal brain slices using a cryostat microtome (Thermo scientific, USA) and collected in PBS. Collected slices were rinsed in PBS 3 times, and incubated for 1hr 30 min with blocking solution (0.3% Triton-X, 2% normal goat serum, 2% normal donkey serum in 0.1 M PBS). Sections were incubated overnight in a mixture of the following primary antibodies with blocking solution at 4°C on a shaker; rabbit anti- Parvalbumin antibody (1:500, Abcam, UK), rabbit anti-mCherry antibody (1:500, Abcam, UK), rabbit-anti-mouse bestrophin antibody, chicken anti-GFP antibody (1:500, Abcam, UK). After washing three times in PBS, sections were incubated with secondary antibodies; conjugated Alexa 405 donkey anti rabbit IgG (1:200, Jackson Immuno Research Laboratories, USA), conjugated Alexa 647 donkey anti Guinee pig IgG (1:200, Jackson Immuno Research Laboratories, USA), conjugated Alexa 594 donkey anti rabbit IgG (1:200, Jackson Immuno Research Laboratories, USA), conjugated Alexa 488 donkey anti chicken IgG (1:200, Jackson Immuno Research Laboratories, USA), for 1hr 30min, followed by three time rinses in PBS. And tissues were counterstained with DAPI (1:3000, Pierce, USA). They are mounted with fluorescent mounting medium (Dako, Denmark). A series of fluorescence images were obtained with Nikon A1 confocal microscopy.

Cell counting

Immunolabeled slices from 2 mice were analyzed for cell counting. Cells were visually inspected and manually counted. Neuronal cells are only counted. When counting cells, laser intensity was adjusted to exclude background negative signal for clearly obtaining viral infected cells as well as Best1 signal.

Statistical analysis

Student's t-test [24] was used to analyze the difference of the intensity of Best1 immunoreactivity. All values indicated are mean±SEM (standard error of mean) and the statistical significance is presented with n.s. (not significant) or asterisks: p values of <0.05(*), <0.01(**), and <0.001(***).

RESULT

Construction of pSico-Red

We developed pAAV-Sico-Red (pSico-Red) vector, which has another reporter gene, *mCherry*, outside of loxP-loxP, according to the cloning strategy (Fig. 1a). For cre-dependent gene-silencing, U6-loxP-CMV-GFP-loxP-shRNA sequence from pSico lentiviral vector was amplified by PCR with MluI or BstB1-AflII-BglII-containing specific primers, and then it was cloned into pAAV-MCS vector. To overcome disadvantage of pSico vector, cre-dependent removal of CMV-GFP sequence, additional EF1a promoter and mCherry sequences were sequentially added into MluI site of first cloned pAAV vector through the MluI, BstB1 and AflII restriction enzyme sites (Fig. 1a).

To confirm if the pSico-Red system is working, we injected pSico-Red vector including Best1 or scrambled shRNA into TRN of DLX5/6-cre mice (Fig. 1b and c). We expected that in terms of the cells infected by pSico-Red Best1 shRNA, cre-negative cells would show both mCherry and GFP signal and cre-positive cells would show only mCherry signal due to excision of GFP, along with stop codon, hindering the Best1 shRNA expression (Fig. 1c, d, and e). Moreover, cre-positive cells would express decreased Best1 expression, compared to scrambled shRNA, because of the gene-silencing effect of Best1 shRNA (Fig. 1e).

Neuronal Best1 expression in TRN

To test the possibility that Best1 is expressed in TRN region, we first performed immunohistochemistry with Best1 and NeuN antibodies. We, for the first time, found that Best1 is highly expressed in the neurons of TRN, indicated by NeuN-positive signals (Fig. 2). The percentage of TRN neurons expressing Best1 was almost 100%, whereas the neurons in the neighboring nuclei, stria terminalis and fimbria hippocampus, were mostly absent of Best1 immunoreactivity (Fig. 2). In marked contrast, we observed much lower level of Best1 in GFAP positive astrocytes than that in neurons (data is not shown). These results raise a possibility that, in addition to well-known astrocytic role, Best1 might have a neuronal function in the brain.

Characterization of TRN neurons of DLX5/6-cre mouse

Based on the cloning strategy for pSico-Red, we predicted that cre-negative cells infected by AAV virus containing pSico-Red vector would express both mCherry and GFP signals, whereas cre-positve cells would express only mCherry signal due to excision of GFP. To validate these possibilities, we analyzed TRN tissues of DLX5/6-cre mice, injected with AAV-pSico-Red virus using confocal microscope. DLX5/6-cre mouse was utilized due to

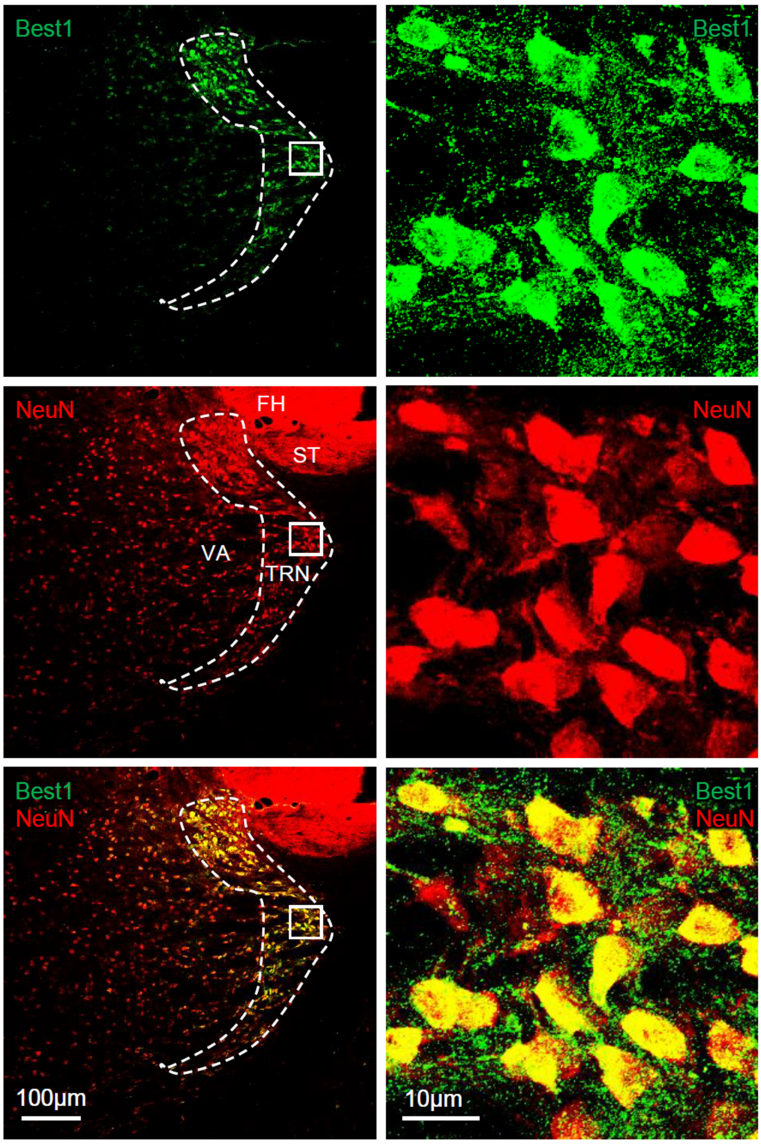

Fig. 2. Neuronal Best1 expression in TRN. Left column shows low magnification images of immunohistochemical analysis. Best1 expression (top), NeuN expression (middle) and their colocalization (bottom). Dashed lines indicate the border of TRN. Abbreviation: TRN, thalamic reticular nucleus; VA, ventral anterior thalamic nucleus; FH, fimbria hippocampus; ST, stria terminalis.

its cell-type expression of cre in a subpopulation of GABAergic interneurons in hippocampus [25, 26]. We observed that most of infected cells in TRN only expressed mCherry signal, implying that these cells are cre-expressing DLX5/6-positive neurons (Fig. 3a and b). However, we also found that a few TRN neurons expressed both mCherry and GFP signals, representing the cre-negative neurons (Fig. 3a and b).

GABAergic interneurons can be subdivided into several classes, including parvalbumin positive neurons [27]. To determine the identity of DLX5/6-cre expressing neurons in TRN, we next performed immunohistochemistry with antibody against parvalbumin (PV). We found that most of the middle- and outer-layer TRN neurons were PV-positive, but not in inner-layer (Fig. 4). Among the mCherry positive neurons (or virus infected neurons) in TRN, about 20% of neurons were PV-positive, and remaining 80% was PV-negative (Fig. 4b). Of the PV-positive and mCherry-positive neurons, 95% turned out to be GFP-positive, which means that these neurons were DLX5/6-negative. In other words, DLX5/6 and PV label two non-overlapping populations of GABAergic interneurons in TRN (Fig. 4b). Taken together, we demonstrate that about 80% of neurons in TRN are DLX5/6-positive and PV-negative GABAergic interneurons, whereas remaining 20% neurons are DLX5/6-negative but PV-positive interneurons.

Cell-type-specific gene-silencing of Best1 in TRN

We have determined that TRN neurons express Best1 and that pSico-Red system identified a subpopulation of TRN neurons. To test the ability of pSico-Red to achieve cell-type-specific

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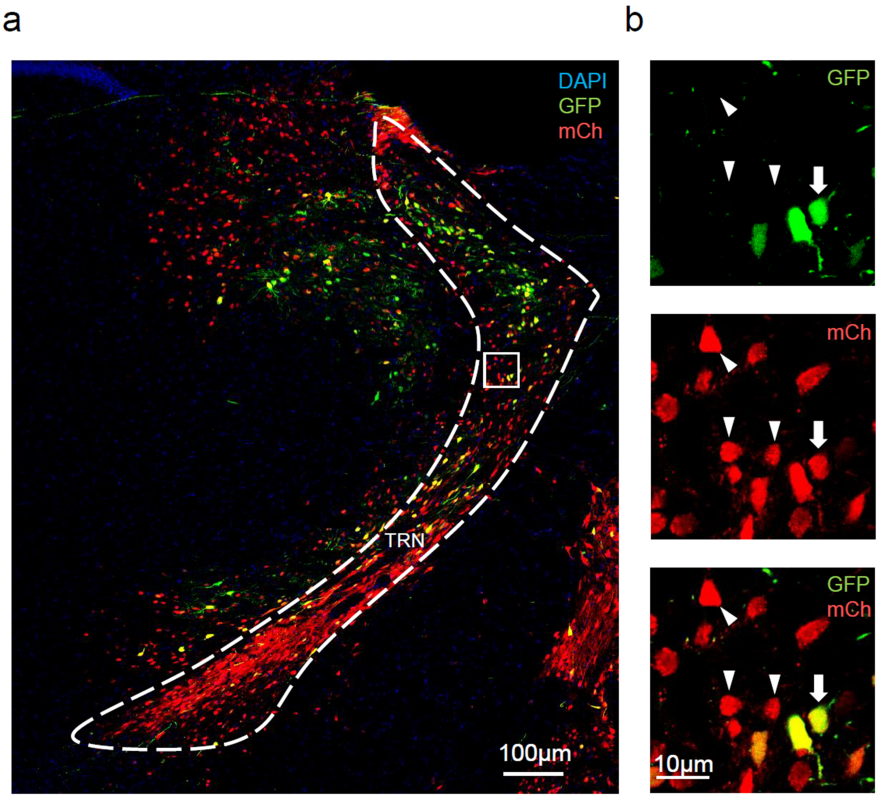

Fig. 3. Cell-type identification of DLX5/6-positive neurons in TRN using pSico-Red virus. (a) Representative image from viral injection of pSico-Red. Dashed line indicates the border of thalamic reticular nucleus (TRN). (b) Magnified images of virus infected neurons from the images in white box. Arrowheads indicate only mCherry$^+$ cell. Arrows indicate mCherry$^+$/GFP$^+$ cell.

gene-silencing, we injected viral pSico-Red that contains either Best1-targeted shRNA or control scrambled shRNA into TRN of DLX5/6-cre mice (pSico-Red-Best1 or pSico-Red-SC). The specificity and efficiency of Best1-targeted shRNA have been reported previously in several reports [3-5, 8]. Then the Best1 immunoreactivity was assessed in mCherry-positive but GFP-negative neurons (Fig. 5a and b). We found that the intensity of Best1 immunoreactivity was significantly decreased in Best1-shRNA group compared to that of scrambled shRNA group (Fig. 5c). These results indicate that pSico-Red is fully functional in cell-type-specific gene-silencing. Furthermore, these results confirm the specificity of Best1 antibody and imply that the immunoreactivity by Best1 antibody is genuinely labeling the Best1 protein.

DISCUSSION

In this study, we have developed advanced gene-silencing system, pSico-Red (Fig. 1). Using pSico-Red, we were able to identify the cells that express shRNA, which was not possible with the original pSico system. In addition, this system allowed us to determine the cell-type classification of cre-expressing neurons in TRN by assessing the distribution pattern of the two fluorescent reporters, mCherry and GFP (Fig. 2 and 3). In addition to these added features, we achieved cell-type-specific gene-silencing with targeted shRNA for Best1. Furthermore, one can combine pSico-Red with viruses carrying promoter specific cre genes in any animal model systems, where cre-expressing animal lines are not available. In this way, cell-type-specific gene-silencing can be achieved even without cre-expressing transgenic animals. In general, most of genes can be silenced in both region-specific and cell-type-specific way by using viruses for pSico-Red in combination with cre-expressing transgenic mice.

The analysis of DLX5/6-cre mouse demonstrated that DLX5/6 defines a new subpopulation of GABAergic TRN interneurons that are non-overlapping and mutually exclusive with PV. Our results showed that there was only 1% of total TRN neurons and 5% of total PV-positive neurons that showed both DLX5/6 and PV expression (Fig. 4b). Previous study reported that about 80% of TRN neurons are somatostatin positive [28], and we found that about 20% of virus infected TRN neurons are PV positive (Fig. 4b). However, it is still unclear what proportion of these neurons are both somatostatin and PV positive. Our results imply that most of DLX5/6 positive neurons are somatostatin positive, given the fact that most DLX5/6 positive neurons are PV negative. Future work is needed to determine the exact extent of overlap between

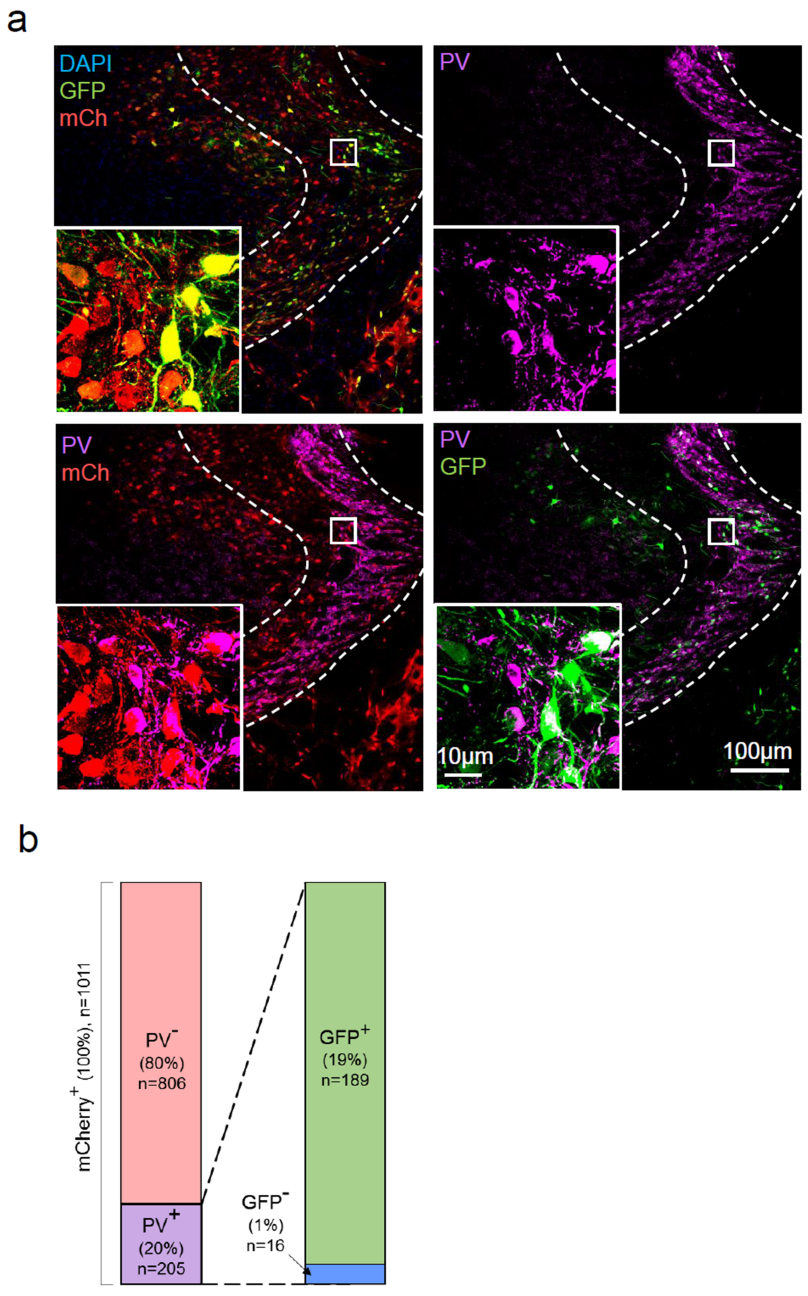

Fig. 4. Analysis of parvalbumin positive neurons in DLX5/6-cre mouse. (a) Merged images of immunohistochemical analysis: DAPI, GFP and mCherry (left-top); PV (right-top); PV and mCh (left-bottom); PV and GFP (right-bottom). Dashed lines indicate the border of TRN. Inset images are magnified images from images in white box of each panel. (b) Quantification of marker positive neurons: $mCherry^+/PV^-$ cells; 79.85±1.81%, $mCherry^+/PV^+/GFP^+$ cells; 18.61±1.12% $mCherry^+/PV^+/GFP$ cells; 1.53±1.12%.

somatostatin and DLX5/6.

We have demonstrated for the first time that Best1 is highly expressed in almost all of TRN neurons, whereas Best1 is weakly expressed in TRN astrocytes. These results raise a possibility that Best1 might have a neuronal function in the brain. In TRN, we raise two potential functions of Best1: tonic GABA release and electrogenic role. Because TRN neurons are GABAergic neurons, Best1 is expected to play an important role in tonic release of GABA. Interestingly, TRN neurons themselves do not express high affinity, extrasynaptic $GABA_A$ receptors that mediate tonic inhibition current [13, 14]. It follows that even if GABA is tonically released through Best1 expressed in TRN neurons, the autocrine action of tonic GABA would be minimal. We have recently reported that tonic GABA in dentate gyrus can inhibit synaptic release of glutamate from perforant fibers via presynaptic $GABA_A$ and $GABA_B$ receptors located in the axon terminals of the perforant fibers [16]. Therefore, we expect that tonic GABA would exert a potent effect on $GABA_A$ and $GABA_B$ that are expressed at presynaptic terminals that terminate onto TRN neurons. Thus, tonic GABA released from TRN neurons would have strong

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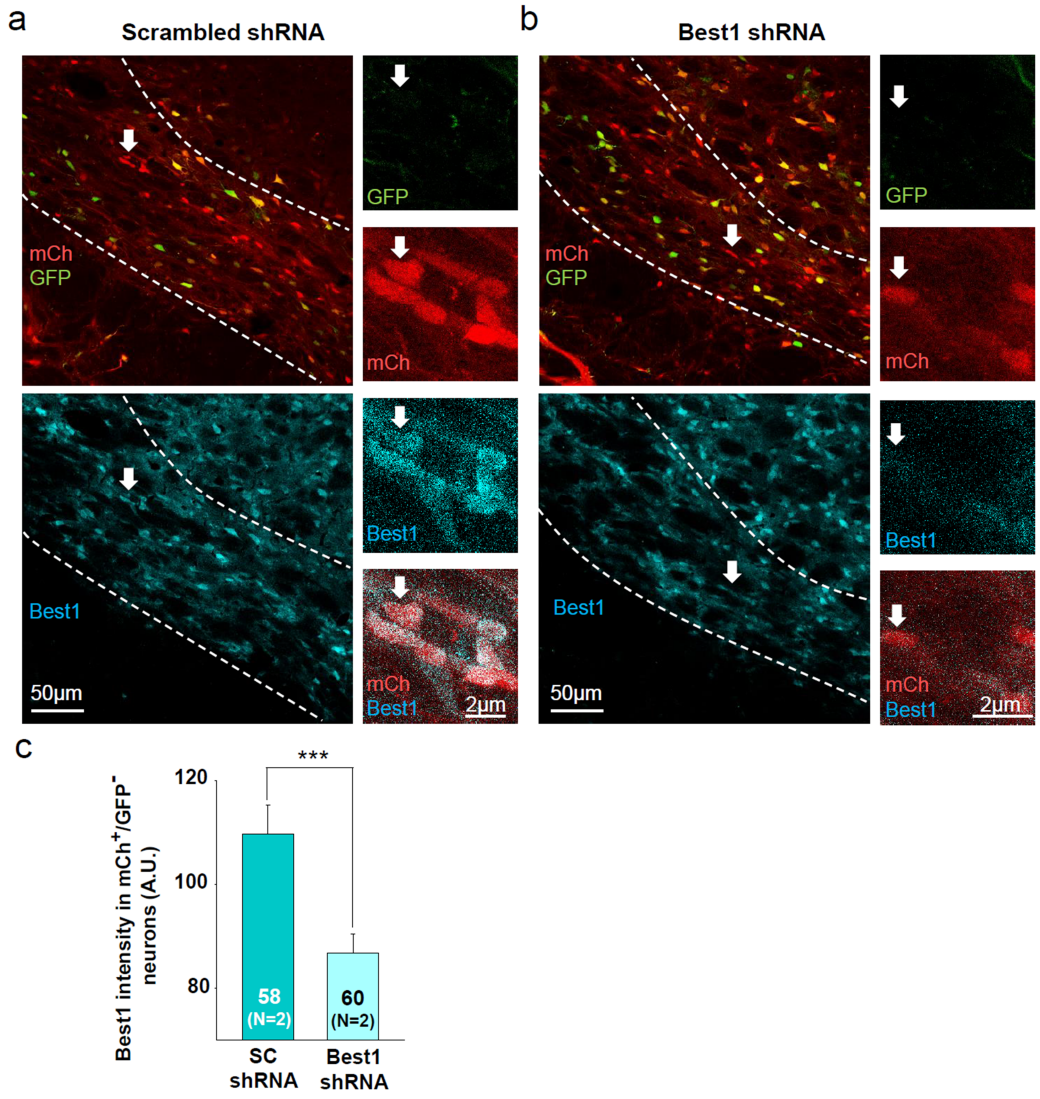

Fig. 5. Cell-type-specific gene-silencing of Best1 in DLX5/6^{+} TRN neurons. (a) Left panel shows low magnification image of TRN with viral infection of pSico-Red scrambled shRNA (top) and Best1 immunostaining (bottom). Upper right panel shows GFP and mCherry fluorescence from infected cell with high magnification (indicated with arrow from left panel). Bottom right panel shows merged image of Best1 and mCherry signal. (b) Left panel shows low magnification image of TRN with viral infection of pSico-Red Best1 shRNA (top) and Best1 immunostaining (bottom). Upper right panel shows GFP and mCherry fluorescence from infected cell with high magnification (indicated with arrow from left panel). Bottom right panel shows merged image of Best1 and mCherry signal. Dashed line indicates the border of TRN. (c) Summary bar graph shows statistical analysis of intensity of Best1 immunoreactivity in mCherry^{+}/GFP^{-} cells (A.U.) in scrambled shRNA group and Best1 shRNA group. Each bar represents the mean±SEM; Student's t-test p values of < 0.001(***). Scrambled shRNA group versus Best1 shRNA group. Number of counted cells for analysis is inserted in each bar. N in parentheses are number of mice.

inhibitory role on presynaptic release of various transmitters including glutamate, in particular, released onto TRN neurons. For example, major projection of cortical neurons to TRN neurons is glutamatergic and this projection could be heavily controlled by tonic GABA release from TRN neurons. Future studies are needed to test these possibilities.

In addition to the possible role of Best1 in tonic GABA release, Best1 could have an electrogenic role in TRN neurons. TRN neurons express high level of voltage-gated Ca^{2+} channels, including Cav3.2 and Cav3.3, which play a critical role in firing pattern and in absence seizure [29]. Because Best1 is a Ca^{2+}-activated chloride channel, Best1 can be activated upon activation of voltage-gated Ca^{2+} channels. In addition to Ca^{2+}-activated potassium channels, such as SK1 and SK2, Best1 could control neuronal excitability, action potential shape and time course, and rhythmic burst discharges of TRN neurons. Future studies are

needed to test these exciting possibilities.

In summary, the present study provides technical advancement in gene-silencing molecular tool by developing pSico-Red, and first evidence for neuronal expression of Best1. In addition, we have identified a novel subpopulation of DLX5/6 expressing, PV-negative GABAergic interneurons in TRN. The function of neuronal Best1 in TRN remains to be explored, but the results and pSico-Red described here should provide an opportunity to address many interesting questions regarding the role of neuronal Best1 in brain function.

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