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Simultaneous neuron- and astrocyte-specific fluorescent marking

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ABSTRACT

Systematic and simultaneous analysis of multiple cell types in the brain is becoming important, but such tools have not yet been adequately developed. Here, we aimed to generate a method for the specific fluorescent labeling of neurons and astrocytes, two major cell types in the brain, and we have developed lentiviral vectors to express the red fluorescent protein tdTomato in neurons and the enhanced green fluorescent protein (EGFP) in astrocytes. Importantly, both fluorescent proteins are fused to histone 2B protein (H2B) to confer nuclear localization to distinguish between single cells. We also constructed several expression constructs, including a tandem alignment of the neuron- and astrocyte-expression cassettes for simultaneous labeling. Introducing these vectors and constructs *in vitro* and *in vivo* resulted in cell type-specific and nuclear-localized fluorescence signals enabling easy detection and distinguishability of neurons and astrocytes. This tool is expected to be utilized for the simultaneous analysis of changes in neurons and astrocytes in healthy and diseased brains.

1. Introduction

Recent neuroscience research has largely focused on the analysis of changes in specific subtypes of brain cells. This focus has led to many insights into the structural alterations in central nervous system disorders, including major depression, Huntington's disease, an autism [1–4]. These findings strongly suggest that many psychiatric diseases feature a major disruption in the normal composition of brain cell populations. It is therefore important to comprehensively investigate which cell types are affected in disease states; however, techniques for simultaneously labeling and imaging of multiple cell types have not yet been adequately developed. In the central nervous system, neurons and astrocytes are likely to be the most affected cell types, making them interesting targets for more detailed analysis.

Cell type-specific marker mice are already widely used and available for a variety of cell subsets, for example EGFP-marker mice are available from the GENSAT project [5]; however, the fluorescent markers expressed by these reporter lines all have the same color and only allow analysis of a single cell type at a time. To investigate distinct cells or cell types at the same time, multicolor labeling systems have also been developed. The Brainbow

Abbreviations: EGFP, enhanced green fluorescent protein; GHE, GfaABC$_1$D-H2B-EGFP; GFAP, glial fibrillary acidic protein; H2B, histone 2B protein; hSyn, human synapsin 1; hSyn-HT, hSyn-H2B-tdTomato; MAP2, microtubule-associated protein 2; NRSE, neuron-restrictive silencer element; PBS, phosphate-buffered saline; Scg10, superior cervical ganglion 10; SNHT, Scg10-NRSE-H2B-tdTomato; SNHT-ins-GHE-ins, Scg10-NRSE-H2B-tdTomato-ins-GfaABC$_1$D-H2B-EGFP-ins.

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mouse is based on the Cre recombinase-controlled expression of a random combination of fluorescent proteins, resulting in Cre-expressing cells that are labeled with various different colors [6]. This system can distinguish individual neighboring cells by their color; thus it is excellent for neuronal tracing approaches but is not applicable for encoding certain cell types by defined colors. A Prism mouse line aims to fulfill this purpose by causing the specific expression of yellow, cerulean, and red fluorescent proteins in oligodendrocytes, astrocytes, and neurons, respectively [7]; however, this transgenic mouse shows an abnormal hyperactive behavioral phenotype due to the overexpression of a nonspecific gene in one of the bacterial artificial chromosomes used for the transgenic mouse generation, rendering this mouse difficult to use as a wild-type reference for psychiatric disease models. Moreover, the fluorescent proteins in the Prism mice and those in most other marker mouse lines are expressed in the cytoplasm; thus, the distinguishability of neurons from their surrounding neighbors in brain areas with highly dense cell populations is difficult. One possible solution to this problem is the targeting of fluorescent signals to the cell nucleus, which makes easy position assignment possible.

In the present study, we attempted to develop a cell type-specific method of marking neurons and astrocytes with nuclear restricted fluorescence signals.

2. Materials and methods

2.1. Vector construction

The constructed cell type-specific expression vectors are shown in Fig. 1. The-116 bp fragment of a neuron-restrictive silencer element (NRSE) fragment and the corresponding complementary sequence were synthesized (Epoch Life Science Inc., TX, USA; sequence in Table S1), annealed, and used as an insert for ligation into the *Bam*HI site of the ptdTomato-N1 vector (Clontech Laboratories Inc., CA, USA) with the In-Fusion HD cloning kit (Clontech Laboratories Inc.). The-2007 bp sequence of a superior cervical ganglion 10 (Scg10) promoter was amplified from rat genomic DNA with the sense primer 5′-GAGGACAATGGA AGTTGTGTTG-3′ and the antisense primer 5′-TTTAGCCATTGTAGG GATGTG-3′, then digested with *Bgl*II and *Sac*II and inserted into the NRSE-containing ptdTomato-N1 vector at the corresponding restriction sites to yield scg10-NRSE-tdTomato. The H2B sequence was excised from pCAG-H2B-tdiRFP-IP (a gift from Dr. Michiyuki Matsuda, Laboratory of Bioimaging and Cell Signaling, Graduate School of Biostudies, Kyoto University) using *Bgl*II and *Bam*HI and inserted into the *Bam*HI site between the NRSE sequence and the tdTomato start codon of scg10-NRSE-tdTomato to yield Scg10-NRSE-H2B-tdTomato (SNHT).

The 681-bp sequence of a compact glial fibrillary acidic protein (GFAP) promoter (GfaABC$_1$D) and the corresponding complementary sequence were synthesized (Epoch Life Science Inc.; Table S1), annealed, and inserted into the *Pac*I and *Xho*I sites of the pEGFP-N1 vector (Clontech Laboratories Inc.) to yield GfaABC$_1$D-EGFP. Then, the H2B sequence was inserted into GfaABC$_1$D-EGFP in the same manner as described above to generate GfaABC1D-H2B-EGFP (GHE). The 5 × tandem insulator sequence (ins [8]) and the corresponding complementary sequence were synthesized, annealed, and inserted into the *Kpn*I and *Sac*I sites of pBluescript II KS(+) (Stratagene, CA, USA) to yield ins-pBluescript, which was then re-digested using *Sal*I and *Spe*I for subsequent insertion upstream of GfaABC$_1$D in GfaABC$_1$D-H2B-EGFP to yield ins-GfaABC$_1$D-H2B-EGFP. A four-part In-Fusion ligation was performed with the combined ins-GfaABC$_1$D-H2B-EGFP cassette excised by *Spe*I and *Afl*II, two linker fragments synthesized as short double-stranded fragments (Epoch Life Science Inc.; Table S1), and the ins-pBluescript vector linearized using *Afl*II and *Not*I, resulting in the GfaABC$_1$D-H2B-EGFP cassette flanked by an insulator sequence on either side to yield the ins-GfaABC$_1$D-H2B-EGFP-ins-pBluescript. Finally, the Scg10-NRSE-H2B-tdTomato cassette excised using *Nhe*I and *Afl*II from SNHT was inserted into the corresponding sites upstream of the first insulator sequence in ins-GfaABC$_1$D-H2B-EGFP-ins-pBluescript to generate Scg10-NRSE-H2B-tdTomato-ins-GfaABC 1D-H2B-EGFP-ins (SNHT-ins-GHE-ins). The human Synapsin 1 (hSyn) promoter sequence was excised from the hSyn-GFP-pLS vector (a gift from Dr. Tohru Matsuki at the Institute for Developmental Research, Aichi Human Service Center) using *Xba*I and *Eco*RI and inserted into the multicloning site of pBluescript to yield hSyn-pBluescript. The H2B-tdTomato cassette from Scg10-NRSE-H2B-tdTomato was inserted into hSyn-pBluescript using the In-Fusion HD cloning kit to generate hSyn-H2B-tdTomato-pBluescript (hSynHT).

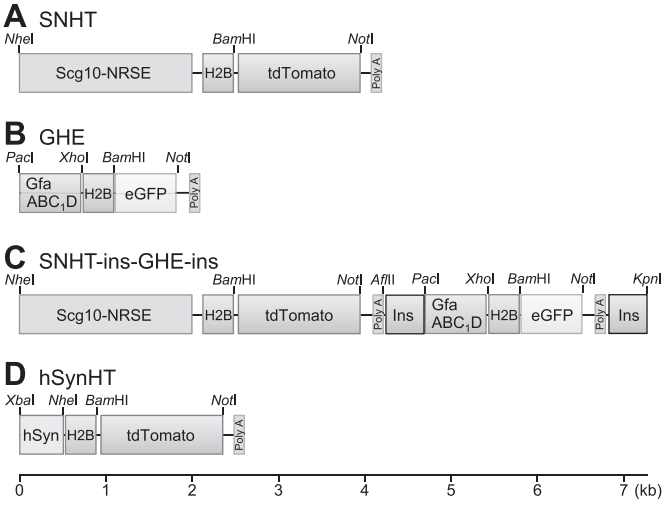

Fig. 1. Schematic construction of cell type-specific expression vectors. A single expression vectors with the neuron-specific promoter Scg10-NRSE (SNHT) (A), a single expression vectors with the astrocyte-specific promoter GfaABC1D (GHE) (B), a tandem expression cassette vector with Scg10-NRSE and GfaABC1D (SNHT-ins-GHE-ins) (C), and a single expression vector with the neuron-specific promoter hSyn (hSynHT) (D) were designed. Nuclear localization was achieved by fusing the fluorescent proteins to the histone 2B (H2B) protein. The restricted digestion sites used for cloning are indicated above the respective vectors. ins, insulator sequence; poly A, polyadenylation site from Simian Virus 40.

2.2. Cloning of lentivirus vectors

The human Synapsin 1 (hSyn) promoter sequence was excised from the hSyn-GFP-pLS vector using *Xba*I and NheI and inserted into the multicloning site of the RFP-QM512B vector from the SpaQ Cumate Switch system (System Biosciences, CA, USA) to generate hSyn-RFP-QM512B. The RFP sequence was then exchanged with the H2B-tdTomato cassette from Scg10-NRSE-H2B-tdTomato by subcloning using the In-Fusion HD cloning kit for site-specific integration to generate hSyn-H2B-tdTomato-QM512B.

The GfaABC$_1$D-H2B-EGFP cassette was first amplified by PCR with the sense primer 5′-GTCACTCGTTTAATTAAGAACATATCC TGGTGTGG-3′ and the antisense primer 5′- GTAATACGACTCACTA-TAGGGC-3′ to add a *Spe*I restriction site upstream of GfaABC$_1$D. The amplified product was then digested using *Spe*I and *Not*I and inserted in place of hSyn-H2B-tdTomato at the corresponding sites of the hSyn-H2B-tdTomato-QM512B vector to generate GfaABC1D-H2B-EGFP-QM512B.

2.3. Electroporation of primary hippocampal cells

Primary cultures of hippocampal cells were prepared as described [9]. The cells at 14 days *in vitro* were transfected with Scg10-NRSE-H2B-tdTomato or GfaABC$_1$D-H2B-EGFP by electroporation with a 4D-nucleofector Y (LONZA Japan Ltd., Tokyo, Japan) according to the Nucleofector Y kit protocol. Briefly, 17.5 μg of purified plasmid DNA was mixed with AD1 nucleofector Y solution and Supplement 1, and then added to the cells from which medium had been removed previously. After electroporation using the nucleofector program EH158 (optimized for primary neuron transfection) the original primary cell medium was immediately placed back onto the cells and the samples were incubated for 3 days before fixation and immunostaining.

2.4. Lentivirus production, concentration, and titration

Briefly, 4 μg of hSyn-H2B-Tomato-QM512B or GfaABC1D-H2B-EGFP-QM512B was transfected with 20 μg of mixed shuttle constructs into Lenti-X 293T cells (Clontech Laboratories Inc.) in 10 cm dishes using polyethylenimine (Polyethylenimine "Max", Mw 40,000; Polysciences, FL, USA). After 12 h of incubation, the culture supernatant was changed to fresh Dulbecco's modified Eagle's medium (DMEM) with 5% GlutaMAX (Sigma–Aldrich, Tokyo, Japan). After 36 h of incubation, the culture supernatant was collected. Lentiviral vector-containing media were filtered through a 0.45 -μm PVDF filter and ultracentrifuged at 23,000 rpm using an SW-28 rotor (Beckman–Coulter, Inc., CA, USA) for 2 h. After ultracentrifugation, the supernatant was removed and the precipitates were dissolved in 30 μl of sterile phosphate-buffered saline (PBS). Lentiviral vectors were aliquoted and stored at −80 °C. The titer of the lentiviral vector solution was estimated using the Global UltraRapid Lentiviral Titer Kit (System Bioscience, CA, USA).

2.5. Lentiviral infection of primary hippocampal cells

First, 0.6 μl of lentivirus harboring hSyn-H2B-tdTomato-QM512B (multiplicity of infection, 0.9) and/or 0.3 μl of lentivirus harboring GfaABC$_1$D-H2B-EGFP-QM512B (multiplicity of infection, 4.6) was added to primary hippocampal cells grown in a 24-well dish at 14 days *in vitro*; then the mixture was incubated, and the medium was changed the next day. After incubation for a total of 8 days, then cells were fixed and immunostained.

2.6. Lentivirus injection into adult mouse brain

Eight-week-old male mice were anesthetized by an intraperitoneal injection of 50 mg/kg pentobarbital. A hole was created in the skull above the PFC at +0.19 mm anterior and −0.05 mm lateral to the bregma. A needle attached to a 25 μl Hamilton syringe was inserted to a depth of 0.5 mm in the prefrontal cortex using stereotactic guidance, and 0.5 μl of the lentiviruses harboring either hSyn-H2B-tdTomato-QM512B or GfaABC$_1$D-H2B-EGFPQM512B was injected into the brain over a period of 5 min. At the end of the inoculation, the needle was left in place for 5 min and then slowly withdrawn. The wound was sutured, and the mice were placed back into their home cages for 10 days before brain extraction. After inoculation for 8 days, the mice were deeply anesthetized with 50 mg/kg pentobarbital, and perfused transcardially with saline followed by 4% paraformaldehyde in PBS. Whole brains were dissected and postfixed in the same fixative overnight at 4 °C. Then, brain blocks were cryoprotected in 20% sucrose in PBS for 48 h at 4 °C. Coronal brain sections (30-μm-thick) were prepared and photographed using a fluorescence microscope (Biozero BZ-9000; Keyence, Osaka, Japan). All animal care and handling procedures were performed in accordance with protocols approved by the Animal Care and Use Committee of the Graduate School of Pharmaceutical Sciences, Osaka University.

2.7. Immunostaining of primary hippocampal cells

Primary hippocampal cells were fixed by 4% paraformaldehyde incubation for 10 min at room temperature. The cells were then washed with PBS and incubated in 0.2% TritonX-100 in PBS for 5 min. After three washes in 0.2% TritonX-100 in PBS, the cells were incubated for 2 h at room temperature in primary antibody solution containing rabbit anti-microtubule-associated protein 2 (MAP2) 1:500 dilution; Chemicon, MA, USA) or mouse anti-GFAP (1:200 dilution; Sigma–Aldrich) antibody in 0.2% TritonX-100 in PBS with 3% bovine serum albumin. After three washes with 0.2% TritonX-100 in PBS, the cells were incubated for 30 min at room temperature with secondary antibody solution containing anti-mouse or anti-rabbit IgG antibody coupled to Alexa-594 (red, 1:1000 dilution; Invitrogen, OR, USA) and anti-mouse or anti-rabbit IgG antibody coupled to Alexa-488 (green, 1:1000 dilution; Invitrogen) in 0.2% TritonX-100 in PBS with 3% bovine serum albumin. After three final washes with 0.2% TritonX-100 in PBS, the cells were mounted on glass slides with Fluoromount (Diagnostic BioSystems, CA, USA) and dried before imaging.

3. Results

3.1. Construction of cell type-specific fluorescent protein expression vectors

We designed expression vectors for cell type-specific labeling of neurons and astrocytes with two distinct colors of fluorescence. For neuron labeling, we chose the pan-neuronal Scg10 promoter, which was reported to be highly neuron-specific in a gene expression analysis of the three major brain cell classes [10], with additional two copies of an NRSE to increase the neuron specificity by inhibiting the promoter activity in non-neuronal cells [11]. However, because the activity of Scg10 promoter was reported to be low in adult brains, we constructed a parallel set of neuron-specific targeting vectors using the well-characterized hSyn promoter [12]. For pan-astrocyte expression, a short 681-bp version of the astrocyte-specific GFAP promoter, GfaABC$_1$D [13], was used because it shows two-fold greater activity than that of the original GFAP promoter and because its small size is advantageous for lentivirus construction, in which the vector size is limited to approximately 10 kb.

We used the enhanced green fluorescent protein (EGFP; [14]) for astrocyte labeling and tdTomato [15], a red fluorescent protein, for neuron labeling because these two colors have clearly separable excitation and emission properties, thus enabling simultaneous imaging of both colors in the same sample. Red fluorescent proteins generally have weak signal intensities, the fluorescent protein often aggregates intracellularly [16]; however tdTomato shows extremely high fluorescence levels due to its dimer conformation, and to date, tdTomato has not been shown to cause aggregation artifacts. EGFP is a well-characterized strong fluorescent protein. Importantly, nuclear localization was achieved by fusing the fluorescent proteins to the histone 2B (H2B) protein, which is a small protein that is transported to the cell nucleus and confers the same intracellular localization to fusion constructs [17].

The various expression vectors were generated by standard cloning procedures (Fig. 1). First, we created separate constructs containing only a single cell type-specific expression cassette, Scg10-NRSE-H2B-tdTomato (SNHT), hSyn-H2B-tdTomato (hSyn-

HT) and GfaABC$_1$D-H2B-EGFP (GHE), to be used in combination for co-transfection or co-injection experiments. Then, we generated a tandem cassette construct, Scg10-NRSE-H2B-tdTomato-ins-GfaABC$_1$D-H2B-EGFP-ins (SNHT-ins-GHE-ins), with both the neuron-specific and astrocyte-specific expression cassettes in the same vector to ensure that the transgenic sequences would be integrated into the same cell. Because the tandem alignment of the two different promoters might lead to read-through into the downstream expression cassette, insulator sequences consisting of five tandem repeats of an insulator element derived from the chicken β-globin gene [8] were inserted flanking the GfaABC$_1$D expression cassette to block read-through and nonspecific fluorescent protein expression [18].

3.2. Expression of cell type-specific markers in dissociated primary hippocampal cells

Cell type-specific expression was tested by transfecting primary hippocampal cell cultures with the neuron-specific and astrocyte-specific labeling vectors (Fig. 2). Because primary hippocampal cell cultures contain both neurons and astrocytes, immunostaining with antibodies against MAP2, a neuron-specific protein, or GFAP, an astrocyte-specific protein, revealed whether the cells showing fluorescent protein expression were the intended target cell type. As shown in Figs. 2A and B, in primary cells transfected with SNHT, tdTomato-expressing cells were also stained green by the anti-MAP2 antibody. We could not detect tdTomato expression in astrocytes in the same dishes, indicating neuron-specific expression. The same results were observed for the second neuron-specific promoter, hSyn (Fig. 2G and H). Correspondingly, in GHE-transfected cells, the EGFP expression colocalized with the GFAP-positive cells, revealing astrocyte-specific expression (Fig. 2C and D), and the transfection of primary cells with the tandem expression construct only induced the expression of tdTomato (Fig. 2E) and EGFP (Fig. 2F) in the respective target cell type. Importantly, for all vectors, the fluorescent signal was correctly targeted exclusively to the cell nucleus. The total number of cells expressing fluorescent protein from the transgene construct was low in all samples due to the low efficiency of electroporation for primary cell transfection (data not shown).

3.3. Lentivirus-mediated expression of cell type-specific markers

For direct injection and labeling of neurons and astrocytes in mouse brains we constructed lentiviral expression vectors. Due to the size restrictions of lentiviral vectors, viruses containing the short single-promoter cassettes hSyn-HT or GHE were generated. Both the infection of single lentiviruses (Fig. 3A–F) and the co-infection (Fig. 3G–I) into primary hippocampal cell cultures resulted in strong EGFP and tdTomato expression in the distinct target cell populations, and the expression was completely restricted to the cell nuclei. For injections into the prefrontal cortex of a wild-type adult mouse brain that were analyzed 1 week later, nucleus-restricted expression of both fluorescent proteins in distinct cells could be observed as well (Fig. 4). This finding confirms the capability of the hSyn and GfaABC$_1$D promoters to drive fluorescent protein expression *in vivo* in the mouse brain and allows for the identification of neurons and astrocytes by the color of their fluorescence in images of the labeled brain region.

4. Discussion

We constructed neuron- and astrocyte-specific expression vectors, which label the nuclei of their respective target cells with red

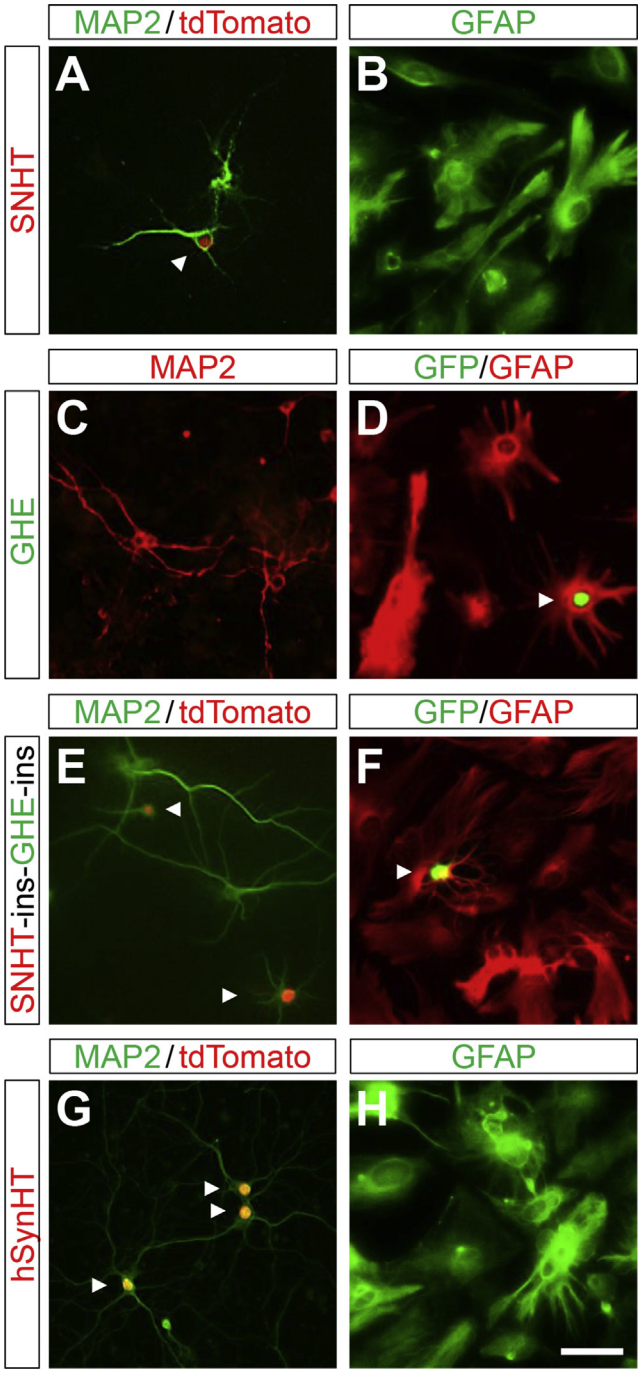

Fig. 2. Neuron- and astrocyte-specific expression of vectors in primary hippocampal cells. Scg10-NRSE-H2B-tdTomato (SNHT) (A and B), GfaABC1D-H2B-EGFP (GHE) (C and D), Scg10-NRSE-H2B- tdTomato-ins-GfaABC1D-H2B-EGFP-ins (SNHT-ins-GHE-ins) (E and F), and hSyn-H2B-tdTomato (hSynHT) (G and H) were electroporated into primary hippocampal cells with 4D-nucleofector Y. After 3 days of incubation, the cells were subjected to immunostaining for the neuron-specific marker protein MAP2 (A, E, and G, green; C, red) and astrocyte-specific marker protein GFAP (D and F, red; B and H, green). Arrowheads indicate transfected cells. Scale bar, 50 μm.

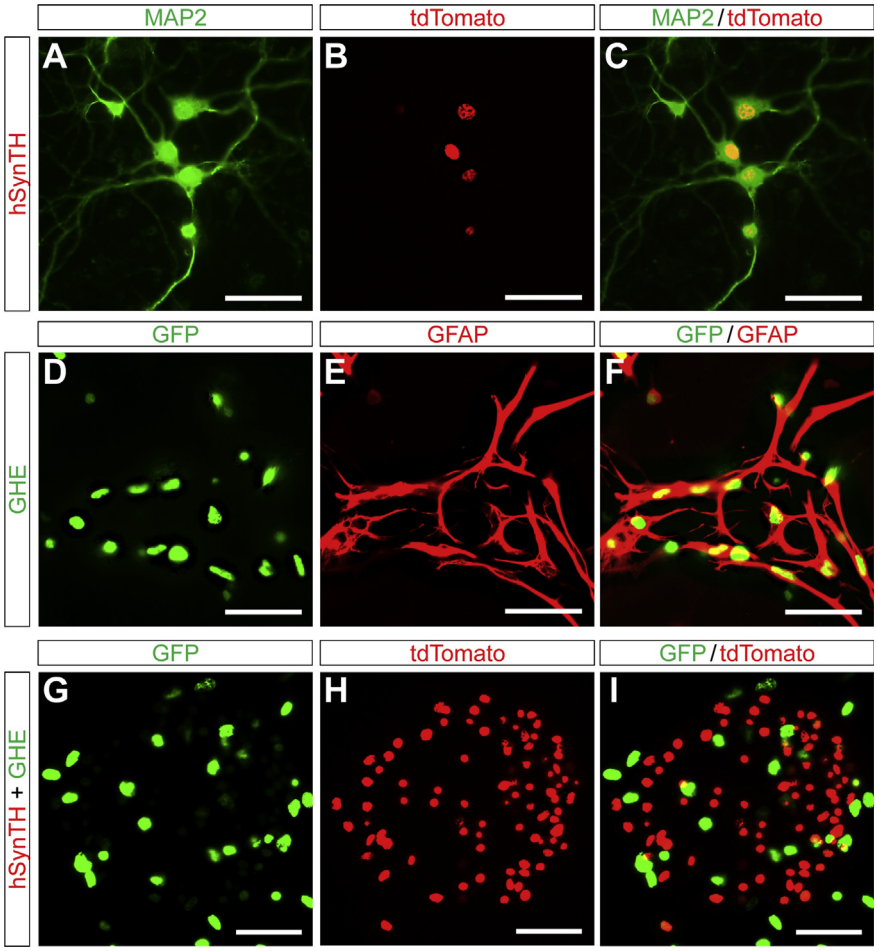

Fig. 3. Lentiviral transduction of primary hippocampal cells. The lentiviral vector hSyn-H2B-tdTomato-QM512B (hSynHT) (A–C) or GfaABC1D-H2B-EGFP-QM512B (GHE) (D–F) alone or their combination (hSynHT + GHE) (G–I) was infected into primary hippocampal cells at 21 days *in vitro*. After 8 days of incubation, the cells were subjected to immunostaining for MAP2 (A and C, green) and GFAP (E and F, red). Scale bars, 50 μm.

or green fluorescent proteins, and we confirmed their utility in the mouse brain using lentiviral injection. This system can be utilized for simultaneous quantitative analysis of the two main cell types in the brain, leading to differentiated information about morphological changes in neuropsychiatric or neurodegenerative diseases.

The two different neuronal promoters that were used, hSyn and Scg10-NRSE, have different expression characteristics and can be chosen depending on the intended application. While hSyn has stronger expression and is more widely used for neuron targeting, it has been reported to show an expression bias for specific neuron subtypes, for example, hSyn favors vertical interneurons over Purkinje cells [19]. This characteristic leads to a more restricted subset of neuron that is labeled and can be analyzed. The Scg10-NRSE promoter, in contrast, is optimized for ubiquitous expression in neurons, and although the expression levels are weaker, the strong intrinsic fluorescence of the tdTomato protein allows for sufficient detection of the fluorescent signal. For astrocyte labeling, it is possible to use promoters of the aldehyde dehydrogenase 1 family member L1, aquaporin 4, connexin 30, GLAST, S100β, or GFAP [20]. Among these, we selected the GFAP promoter ($GfaABC_1D$) for lentiviral vectors because of both its small size and its greater activity.

In addition to neurons and astrocytes, the brain consists of many other cell types such as oligodendrocytes or microglia. Additionally, among neurons, functionally distinct excitatory or inhibitory subtypes using different neurotransmitters (e.g., glutamatergic, dopaminergic, or serotoninergic neurons) are known to play specific roles in psychiatric diseases [21]. Thus, for an even more comprehensive analysis of the changes in cell composition in disease phenotypes, it is essential to observe even more classes of cells. This examination can be achieved by using additional virus vectors encoding marker fluorescent proteins in other cell types or by using our neuron- and astrocyte-marker lentiviral vectors in transgenic mouse strains that already show labeling of a specific cell type of interest.

Compared with the approach of other groups using multi-transgenic mice, e.g., the PRISM mouse [7], our lentiviral system has the benefit that the fluorescent marker proteins can be introduced at the specific time point that will be analyzed. This strategy avoids the possible developmental effects caused by the constitutive expression in transgenic mouse lines that can lead to the behavioral phenotypes observed in the PRISM mouse line. Additionally, the viral vector approach is more efficient and flexible considering the time-intensive maintenance and crossbreeding required for transgenic mouse lines.

Conflict of interest

None.

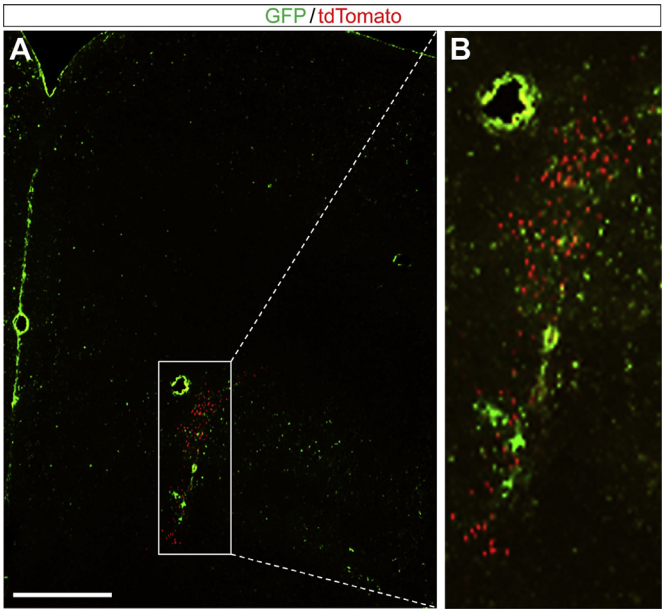

Fig. 4. Lentiviral transduction of the prefrontal cortex of adult mouse brain. hSyn-H2B-tdTomato-QM512B and GfaABC$_1$D-H2B-EGFP-QM512B were co-injected unilaterally into the prefrontal cortex of 8-week-old mouse brains. Eight days after the injection, the section containing the injection site was imaged using fluorescence microscopy. A magnification of the area marked with a white box in (A) is shown in (B). Strong tdTomato (red) and EGFP (green) expression in cell nuclei around the area of injection can be observed. Scale bar, 500 μm.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.073.

Appendix A. Supplementary data

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