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Generation of a MOR-CreER knock-in mouse line to study cells and neural circuits involved in mu opioid receptor signaling

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Abstract

Mu opioid receptor (MOR) is involved in various brain functions, such as pain modulation, reward processing, and addictive behaviors, and mediates the main pharmacologic effects of morphine and other opioid compounds. To gain genetic access to MOR-expressing cells, and to study physiological and pathological roles of MOR signaling, we generated a MOR-CreER knock-in mouse line, in which the stop codon of the *Oprm1* gene was replaced by a DNA fragment encoding a T2A peptide and tamoxifen (Tm)-inducible Cre recombinase. We show that the MOR-CreER allele undergoes Tm-dependent recombination in a discrete subtype of neurons that express MOR in the adult nervous system, including the olfactory bulb, cerebral cortex, striosome compartments in the striatum, hippocampus, amygdala, thalamus, hypothalamus, interpeduncular nucleus, superior and inferior colliculi, periaqueductal gray, parabrachial nuclei, cochlear nucleus, raphe nuclei, pontine and medullary reticular formation, ambiguous nucleus, solitary nucleus, spinal cord, and dorsal root ganglia. The MOR-CreER mouse line combined with a Cre-dependent adeno-associated virus vector enables robust gene manipulation in the MOR-enriched striosomes. Furthermore, Tm treatment during prenatal development effectively induces Cre-mediated recombination. Thus, the MOR-CreER mouse is a powerful tool to study MOR-expressing cells with conditional gene manipulation in developing and mature neural tissues.

Keywords

Mu opioid receptor, striosome, Cre recombinase, knock-in mouse, *in situ* hybridization

1 Introduction

Mu opioid receptor (MOR), a G-protein coupled receptor (GPCR) encoded by the *Oprm1* gene, mediates physiological actions of endogenous opioid peptides, and is critically involved in regulating pain, reward processing, and addictive behaviors (Le Merrer, Becker, Befort, & Kieffer, 2009). MOR signaling has also stimulated much clinical interest because MOR is the primary target of morphine and plays a key role in mediating the pharmacological effects of opioid compounds.

Ligand binding to MOR leads to activation of heterotrimeric G proteins Gi/Go, which have downstream effects on intracellular processes, including inhibition of adenylyl cyclase, gating of ion channels, and activation of mitogen-activated protein kinase pathways (Law, Wong, & Loh, 2000). Cellular and molecular modification of neuronal excitability mediated by MOR signaling has been intensively studied (von Zastrow, & Williams, 2012). However, it remains largely unknown how MOR-mediated neuromodulation induces not only short-term physical or cognitive effects but also long-lasting behavioral changes observed in drug addiction. This is partly because MOR is expressed in discrete populations of neurons throughout the nervous system (Delfs et al., 1994; Kaufman et al., 1995; Mansour et al., 1994; Minami et al., 1994). It is still challenging to precisely identify MOR-expressing cells and dissect their contributions to the brain circuit operation. Toward the understanding of physiological and pathological roles of MOR signaling, genetically engineered animal models to study MOR-expressing cells would be useful.

Furthermore, accumulating evidence indicates that MOR expression in the brain is dynamically changing during prenatal and postnatal development (Tong et al., 2000; Zhu, Hsu, & Pinar, 1998), pointing to a need for temporospatial control of genetic manipulation of MOR-expressing cells in the brain circuit. Thus, with the aim of establishing an inducible genetic system for manipulation of MOR-expressing neurons, we developed a MOR-CreER knock-in mouse line. In this study, we describe the generation of a MOR-CreER strain and characterize

the recombination activity using a Cre-responsive reporter mouse line and adeno-associated virus (AAV) vector system.

2 Results and discussion

To achieve inducible genetic manipulation of MOR-expressing cells *in vivo*, we designed a gene-targeting construct, in which the sequence encoding the Cre recombinase-modified estrogen receptor fusion protein (iCreER^{T2}) (Feil, Wagner, Metzger, & Chambon, 1997; Shimshek et al., 2002) was fused with the *Thosea asigna* virus 2A-like peptide (T2A) sequence (Kim et al., 2011), and we used this cassette to replace the stop codon in exon 4 of the *Oprm1* gene (Figure 1a). The linearized targeting construct was electroporated into C57BL/6 mouse ES cells (Fujihara, Kaseda, Inoue, Ikawa, & Okabe, 2013). A total of 48 neomycin (Neo)-resistant ES cell clones were characterized by PCR, and 24 clones were confirmed to have undergone the expected homologous recombination (Figure 1b). A chimeric mouse derived from one of the clones was crossed with CAG-FLPe mice (Kanki, Suzuki, & Itohara, 2006) to remove the Neo selection cassette in the offspring (Figure 1c). We confirmed germline transmission of the targeted allele, and subsequently bred *inter se* to obtain heterozygous (KI/WT) and homozygous (KI/KI) MOR-CreER mice (Figure 1d).

To assess Tm-inducible Cre recombinase activity, MOR-CreER mice were crossed with ROSA26 reporter (R26-CAG-LoxP-mTFP1) mice, in which HA-tagged mTFP1 (bright teal fluorescent protein) is conditionally expressed after Cre-mediated recombination (Imayoshi et al., 2012). Immunohistochemistry (IHC) of the brain sections was performed using anti-HA tag antibody. A strong mTFP1 reporter signal enhanced by immunolabelling was observed in Tm-treated MOR-CreER^{KI/KI}; R26-CAG-LoxP-mTFP1 mice (Figures 1e and 2), while background, leaky fluorescence was minimal in Tm-untreated mice (Figure 1f). Thus, Cre recombinase activity is tightly regulated by Tm administration. Although MOR expression in glial cells cannot be entirely excluded, no apparent reporter signal was detected on glial cells.

Among the telencephalic structures shown in Figure 2a–i, the olfactory bulb, cerebral

cortex, striatum (also referred to as the neostriatum or caudate-putamen), and dorsal endopiriform nucleus contained areas densely-packed with mTFP1-expressing cells. In the olfaction-related circuits, labeled cells were densely distributed in the granule cell and glomerular layers of the olfactory bulb, and the anterior olfactory area external part (Figure 2a, b). Although mTFP1-expressing cells were observed throughout the cerebral cortex, the primary somatosensory cortex (S1) displayed a clear laminar distribution of labeled cells in layer V and in the deep layer VI (Figure 2e–g). mTFP1-expressing cells were also prominent in the orbitofrontal cortex (Figure 2c), retrosplenial cortex (Figure 2g–i) and dorsolateral entorhinal cortex (Figure 2h, i). In the striatum, mTFP1-expressing cells were concentrated into patchy clusters (Figure 2d–f). These patterns presumably correspond to striosomes (also known as patches), which have been identified as MOR-enriched compartments compared to the surrounding matrix, using opioid binding autoradiography (Pert, Kuhar, & Snyder, 1976) and MOR IHC (Arvidsson et al., 1995; Kaneko, Minami, Satoh, & Mizuno, 1995; Mansour, Fox, Burke, Akil, & Watson, 1995). In the hippocampus, mTFP1-expressing cells displayed a scattered distribution in the CA1, CA3, and dentate gyrus (Figure 2g, h), consistent with previous studies that showed that MOR is mainly expressed in the subpopulation of hippocampal interneurons (Drake & Milner, 2002). In the amygdala and extended amygdala, mTFP1-expressing cells were mainly found in the intercalated nucleus of the amygdala, central and medial nuclei of the amygdala (Figure 2g), the amygdalohippocampal area (Figure 2i), and the bed nucleus of the stria terminalis (Figure 2f).

Among the diencephalic areas, the medial habenula (MHb), which was reported as one of the strongest expression sites for MOR in mice (Gardon et al., 2014), was enriched with the labeled cells (Figure 2g). In addition, mTFP1-expressing cells were also densely distributed in the thalamic nuclei, including the paraventricular, paracentral, central medial, centrolateral, rhomboid and xiphoid nucleus (Figure 2g), the hypothalamic nuclei including the medial preoptic area, medial preoptic nucleus, lateral hypothalamic area (LH) (Figure 2f, g), and the medial mammillary nucleus (Figure 2h).

In the midbrain, mTFP1-expressing cells were densely distributed in the superior and inferior colliculi, periaqueductal gray, and interpeduncular nucleus (Figure 2i, j). In the pons and medulla, labeled cells were densely observed in the external part of the lateral parabrachial nucleus (LPBE), raphe nuclei, dorsal cochlear nucleus, ambiguus nucleus (Amb), and solitary nuclei (Figure 2j-l). In the spinal cord, mTFP1-expressing cells were enriched in the dorsal horn (Figures 2m and S1). As shown in Figures 2m and S1, a subset of primary sensory neurons in the dorsal root ganglia (DRG) expressed the reporter protein.

These findings are consistent with the previously reported *in situ* hybridization (ISH) analyses for *Oprm1* mRNA (Kaufman et al., 1995; Delfs et al., 1994; Mansour et al., 1994; Minami et al., 1994), and immunohistochemical or autoradiographic studies for MOR (Arvidsson et al., 1995; Kaneko et al., 1995; Mansour et al., 1995; Pert et al., 1976).

Next, to confirm the T2A-mediated bicistronic expression of MOR and iCreER^{T2} and to evaluate the Cre-mediated recombination efficiency in the nervous system, we performed combined ISH for *Oprm1* mRNA and IHC for HA-mTFP1 in Tm-treated MOR-CreER^{KI/KI}; R26-CAG-LoxP-mTFP1 mice. As shown in Figures 3 and S2, although some *Oprm1* mRNA-expressing cells were negative for HA-mTFP1 immunoreactivity, most of the *Oprm1* mRNA-expressing cells expressed the reporter mTFP1 two to four weeks after the final Tm administration. *Oprm1* mRNA signals were observed in $98.1 \pm 0.3\%$ (mean $\pm$ SEM, 356 cells in 363 cells analyzed, $n = 3$ mice) of the cells with HA-mTFP1 immunoreactivity in the striatum, $97.4 \pm 1.7\%$ (483/497) in the S1 layer V, $96.6 \pm 1.7\%$ (185/192) in the CA1, $99.3 \pm 0.4\%$ (744/750) in the MHb, $94.9 \pm 3.0\%$ (267/284) in the LH, $99.5 \pm 0.5\%$ (376/378) in the LPBE, $98.7 \pm 0.6\%$ (172/174) in the Amb, and $99.6 \pm 0.2\%$ (451/453) in the DRG. To directly assess the relationship between the iCreER^{T2} expression level and Tm-induced recombination activity, we conducted combined labeling for iCreER^{T2} mRNA and HA-mTFP1 immunoreactivity in Tm-treated MOR-CreER^{KI/KI}; R26-CAG-LoxP-mTFP1 mice. As shown in Figure S3, some iCreER^{T2}-expressing cells were negative for HA-mTFP1 immunoreactivity, but the distribution pattern of iCreER^{T2} mRNA was almost identical to that of *Oprm1* mRNA shown in Figure 3.

These results indicate that the MOR-CreER mouse expresses iCreER^{T2} under the transcriptional control of the *Oprm1* gene without altering its physiological expression, and Tm-induced recombination activity closely recapitulates the expression of *Oprm1* mRNA in the adult nervous system.

Previous studies revealed that striosome and matrix compartments in the striatum are organized differently in many aspects, and striosome neurons serve distinctive cognitive functions in decision-making under conflict (Friedman et al., 2015) and reward prediction (Yoshizawa, Ito, & Doya, 2018). Because the striosomes are histochemically identified based on the expression level of MOR, MOR-CreER mice would be a useful model to target and study MOR-expressing striosome neurons. Toward that end, we performed double labeling for mTFP1 and MOR immunoreactivity in Tm-treated MOR-CreER^{KI/KI}; R26-CAG-LoxP-mTFP1 mice and quantitatively analyzed the mTFP1-positive cell density between the MOR-immunoreactive (ir) striosome and the surrounding matrix. The clusters of mTFP1-positive cells correlated with the MOR-ir striosome compartments (Figure 4a–b”). We measured the cell density in the rostral (bregma +1.21 mm ± 0.12 mm), middle (+0.49 mm ± 0.12 mm), and caudal (–0.23 mm ± 0.12 mm) coronal sections. The densities of mTFP1-positive cells were 7–15 fold higher in the striosomes than in the matrix in the same sections ($p < 0.0001$) (Figure 4c), indicating that the MOR-CreER mediated recombination predominantly occurs in the striosome.

To validate the predominance of Cre recombination activity in the highly MOR-expressing striosome compartments, we tested another Cre-dependent reporter method, an AAV vector containing a double-inverted open reading frame (DIO). We injected AAV9-hSyn-DIO-EGFP into the striatum of MOR-CreER^{KI/WT} mice, followed by a 5-day Tm administration (Figure 5a, b). Again, we found that the pattern of EGFP expression closely recapitulated that of highly MOR-ir striosomes (Figure 5c–d”). Thus, a combination of MOR-CreER mice and locally injected viral vectors permits transgene expression in spatially restricted MOR-expressing cells, and may help to dissect the functions of MOR signaling and MOR-expressing cell populations.

190 Lastly, to examine MOR-CreER mediated recombination during striatal development,
191 we treated MOR-CreER^{KI/KI}; R26-CAG-LoxP-mTFP1 mouse embryos with Tm. Historically,
192 *Oprm1* mRNA was first detected at embryonic day 11.5 (E11.5) in the basal ganglia during
193 development (Zhu et al., 1998). Consistent with this, we observed a cluster of mTFP1-positive
194 cells in the striatum of E12.5 embryos following Tm administration at E11.5 (Figure 6a). mTFP1
195 fluorescence in the striatum was visible in the whole mount embryo at E14.5 following E12.5
196 Tm treatment (Figure 6b). To examine MOR-CreER mediated recombination in late gestation,
197 we injected Tm at E17.5 and analyzed mTFP1 expression at postnatal day 0 (P0).
198 mTFP1-expressing cells were distributed widely in the striatum (Figure 6c). Some cells were
199 observed within the regions immunoreactive for dopamine and cAMP-regulated phosphoprotein
200 (DARPP32) (Figure 6c", d), which marks the striosomes at early stages of striatal development
201 (Passante et al., 2008). In addition, many mTFP1-expressing cells were also found in the
202 surrounding area (Figure 6d). Consistent with this finding, the previous ISH study reported that
203 the signals for *Oprm1* mRNA show a homogeneous pattern in the striatum of E17.5 embryos, in
204 contrast to the patchy distribution in adults (Zhu et al., 1998).

205 It is intriguing that the *Oprm1* mRNA distribution changes from diffuse to patchy
206 during striatal development (Tong et al., 2000). However, it remains unclear whether the
207 diffusely distributed MOR-expressing cells in the prenatal striatum eventually form MOR-ir
208 striosomes or if this MOR expression is transient during the prenatal period. Because the
209 MOR-CreER allele is Tm-inducible, it enables us to label MOR-expressing cells at specific time
210 points during development. Thus MOR-CreER mice might facilitate further examination of the
211 functional maturation of the striosome and matrix compartments.

212 In summary, we generated and characterized a novel inducible Cre knock-in mouse
213 line, MOR-CreER. We showed that recombination activity specific to the MOR-expressing cells
214 is tightly regulated in a Tm-dependent manner, not only in the adult brain but also in the
215 developing brain. In the striatum, MOR-CreER mediated recombination with a ROSA26 reporter
216 and a Cre-dependent AAV vector occurred predominantly in the striosomes, which are identified

as MOR-enriched compartments. Therefore, MOR-CreER mice will be useful to explore this intriguing structure. Because the MOR-CreER allele is Tm-inducible, it also enables timed recombination during development, which will greatly facilitate the study of the prenatal and postnatal development of striatal compartments. Thus, the MOR-CreER mouse line is a powerful new tool to study MOR-expressing neuronal populations.

3 Materials and Methods

3.1 Animals

All experiments conformed to the guidelines of the National Institutes of Health (NIH) experimental procedures and were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University. CAG-FLPe (Kanki et al., 2006), R26-CAG-LoxP-mTFP1 (RBRC05147) (Imayoshi et al., 2012), C57BL/6J, and Slc:ICR mice (Japan SLC, Hamamatsu, Japan) were used. All mice strains used in the histological analysis were maintained in a C57BL/6J background. Mice were housed with food and water available *ad libitum* in light-controlled (12 h light: 12 h dark cycle) mouse facilities. Male mice aged 2–8 months were used for the analyses of the adult stage. For the studies during prenatal development, we administered Tm to pregnant females, and used embryos without distinction of sex.

3.2 Construction of the targeting vector for homologous recombination

A bacterial artificial chromosome (BAC) containing the mouse *Oprm1* gene (RP24-112H16) was obtained from BACPAC Resources Center at Children's Hospital Oakland Research Institute (CHORI). The BAC was modified so that the translational stop codon in exon 4 of the *Oprm1* gene was replaced by a cassette comprising the T2A sequence, iCreER^{T2} gene with stop codon and FRT-flanked neomycin-resistant gene (T2A-iCreER^{T2}-FRT-Neo-FRT), using standard cloning techniques and BAC recombineering (Lee et al., 2001). A retrieving vector was generated by cloning 0.5 kb length homology arms from the gene into the plasmid pMCS-DTA (a gift from Dr. Kosuke Yusa, Osaka University, Japan), which contains the diphtheria toxin

fragment-A (DTA) gene driven by the MC1 promoter for negative selection in ES cells. The homology arms were designed so as to cover regions 2.1 kb upstream and 7.7 kb downstream of the targeting site. The retrieving vector was introduced by electroporation into the recombineering strain EL250 containing the modified BAC DNA, and the final targeting vector was obtained using BAC recombineering. The targeting vector contained the 5' homology arm (2.1 kb), T2A-iCreER^{T2}-FRT-Neo-FRT cassette, 3' homology arm (7.7 kb) and the DTA gene (Figure 1a). The integrity of the targeting vector was confirmed by sequencing and restriction enzyme analysis. The detailed cloning strategy and the complete sequences of the plasmids are available upon request.

3.3 Generation of MOR-CreER knock-in mice

The homologous recombination in the mouse ES cells was performed as reported previously (Imayoshi et al., 2012). The targeting vector was linearized by *AscI* and electroporated into C57BL/6 mouse ES cells, EGR-101 (Fujihara et al., 2013). G418 resistant clones were selected, and candidate clones were screened for homologous recombination by detecting the expected length of PCR products amplified with primers designed to flank the 5' arm and 3' arm, respectively (Figure 1a,b). ES cells from two confirmed clones C13 and C19 were injected into Slc:ICR blastocysts and implanted into pseudopregnant Slc:ICR females to generate chimeric mice. A chimeric mouse derived from clone C13 was crossed with CAG-FLPe mice (Kanki et al., 2006) to remove the FRT-flanked neomycin resistance cassette in the offspring. Genotyping PCR was performed using genomic DNA extracted from tail tips as templates. PCR Primers used were F4 (5'-ATGAGCCCTCAGTACTCACTCTCT-3'), R4 (5'-TCCTGCCAATGTGGATCAGCATTC-3') and R5 (5'-TGACATCTATTTTCCCTTGATGA-3'). Homozygous MOR-CreER mice were born at expected Mendelian ratios; they were viable and fertile without obvious gross abnormalities. MOR-CreER mice will be available to the scientific community upon request.

3.4 Tamoxifen (Tm) treatment

To activate CreER^{T2} recombinase in adult MOR-CreER^{K1/K1}; R26-CAG-LoxP-mTFP1 mice, 125 mg/kg of Tm (T5648; Sigma-Aldrich, St Louis, MO, USA) in corn oil (C8267; Sigma-Aldrich) was intragastrically administered on five consecutive days, followed by one week of recovery. This procedure was repeated for one or two more rounds. Shorter Tm exposure led to lower recombination efficiency but did not clearly change the distribution pattern of labeled cells. For recombination of the Cre-dependent viral vector, 100 mg/kg of Tm was administered on five consecutive days. For recombination in embryos, pregnant dams were intraperitoneally or intragastrically treated once with 4 mg of Tm.

3.5 AAV Preparation and stereotaxic injection

Recombinant AAV was produced as reported previously (Matsui, Tanabe, & Watanabe, 2012) with slight modifications. Briefly, AAV-293 cells (Agilent Technologies, Santa Clara, CA, USA) at approximately 80–90% confluence were co-transfected with pAAV-hSyn-DIO-EGFP (#50457; Addgene, Cambridge, MA, USA), the rep2/cap9-expressing plasmid (Penn Vector Core, Philadelphia, PA, USA) and the pHelper plasmid (Agilent Technologies) by the polyethylenimine method. Cells were harvested 72 hours after transfection and then pelleted by centrifugation at 2,500 ×g for 5 minutes. The pelleted cells were lysed in PBS by a triple freeze-and-thaw procedure. The cell lysate was treated with 4 U/ml of DNase I for 30 minutes at 37°C and centrifuged at 15,300 ×g for 10 minutes. The virus-containing supernatant was further purified by iodixanol (Opti-Prep; Axis-Shield, Oslo, Norway) step-gradient ultracentrifugation, and the solution was concentrated to approximately 200 µl using a VivaSpin 6 unit (100,000 MWCO, Sartorius Stedim Biotech, Aubagne, France). The DNase-resistant viral genome titer was determined by quantitative real-time PCR. The viral solution was stored at –80°C until use.

For stereotaxic injections into the striatum, mice were deeply anesthetized with ketamine and xylazine. A glass pipette with a tip of approximately 50 µm was stereotaxically placed in the striatum (0.8 mm anterior to the bregma, 1.6 mm lateral to the midline, and 2.6 mm

deep from the brain surface), and 1 μ l of viral solution diluted to 2.5×10^{12} viral particles/ml with PBS was injected using a 25 μ l Hamilton syringe connected to a microinfusion pump. Mice were allowed to recover from surgery for two weeks before Tm treatment.

3.6 Tissue preparation

For Tm-treated adult mice, transcardial perfusion was performed 2–6 weeks after the last Tm administration. Mice were deeply anesthetized with sodium pentobarbital and then transcardially perfused with 0.01 M PBS followed by 4% paraformaldehyde (PFA) in 0.1 M PB (pH 7.4). For embryos and P0 pups, mice were deeply anesthetized with hypothermia and then decapitated. Brains were removed and postfixed with 4% PFA in 0.1 M PB overnight at room temperature for IHC, or for 3 days at 4°C for ISH. After cryoprotection with 30% (w/v) sucrose in 0.01 M PBS, blocks of brains of adult mice or brains of P0 pups were cut into 30- μ m-thick sections with a cryostat. DRGs were cut into 50- μ m-thick sections for ISH. Spinal cords and DRGs were cut into 20- μ m-thick sections for IHC. E12.5 embryos were cut into 10- μ m-thick sections.

3.7 Immunofluorescence labeling

Free-floating brain sections were incubated overnight at room temperature with primary antibodies in PBS containing 0.3% (w/v) Triton X-100 (PBS-X) and 10% normal goat serum (NGS). The primary antibodies used were 1:500-diluted rat anti-HA antibody (11867431001; Sigma-Aldrich) for detecting HA-mTFP1, 1:600-diluted rabbit anti-MOR antibody (RA10104; Neuromics, Edina, MN, USA), 1:250-diluted rabbit anti-DARPP32 antibody (2306S; Cell Signaling Technology, Beverly, MA, USA), and 1:500-diluted mouse anti neuronal nuclear antigen (NeuN) antibody (MAB377; Merck Millipore, Burlington, MA, USA). After three washes with PBS, the sections were incubated with secondary antibodies in PBS-X and 0.5% NGS. The secondary antibodies used were Alexa Fluor 488-conjugated goat anti-rat IgG antibody (8 μ g/ml, A-11006; Thermo Fisher Scientific Inc., Waltham, MA, USA), Alexa Fluor 555-conjugated goat anti-rabbit IgG antibody (8 μ g/ml, A27039; Thermo Fisher Scientific Inc.),

and Alexa Fluor 647-conjugated goat anti-mouse IgG antibody (8 µg/ml, A-21235; Thermo Fisher Scientific Inc.). For embryonic sections, nuclei were counterstained with DAPI. For the sections including the spinal cord, DRG, and embryonic brain, IHC was performed on glass slides with the same solutions described above.

3.8 Combined fluorescent labeling for HA immunoreactivity and mRNA signals

Relevant fragments of the *Oprm1* cDNA (nucleotide 682–1502; GenBank accession No. NM_001302793.1) or iCreER^{T2} (41–1066; AY056050.1) were cloned into a pBluescript II SK(+) (Stratagene, La Jolla, CA, USA) plasmid vector. Digoxigenin (DIG)-labeled cRNA probes were synthesized with T3 or T7 RNA polymerase and DIG RNA Labeling Mix (11277073910; Sigma-Aldrich) using linearized plasmids as templates.

Fluorescent ISH combined with fluorescent IHC was performed as reported previously (Hioki et al., 2010). Free-floating sections were treated with 2% (w/v) H₂O₂ followed by 0.3% Triton X-100, and then hybridized for 16–20 hours at 60°C with 1 µg/ml DIG-labeled sense or antisense cRNA probe in hybridization buffer. After washing and ribonuclease A treatment, the sections were incubated overnight at room temperature with 1:4000-diluted horseradish peroxidase-conjugated sheep anti-DIG antibody (11207733910; Sigma-Aldrich), 1:500-diluted rat anti-HA antibody, and 1:1000-diluted mouse anti-NeuN antibody. HA-immunostaining was required because mTFP1 fluorescence was dramatically reduced after hybridization, likely due to heat denaturation (Nakamura, Kameda, Koshimizu, Yanagawa, & Kaneko, 2008). After washing, the sections were incubated with 8 µg/ml Alexa Fluor 488-conjugated goat anti-rat IgG antibody and 8 µg/ml Alexa Fluor 647-conjugated goat anti-mouse IgG antibody for 1 hour in Tris-HCl (pH 7.5)-buffered saline (TBS). The sections were then treated with a TSA-Plus Biotin Kit (PerkinElmer Inc., Waltham, MA, USA) for 20 minutes and finally incubated with 2 µg/ml Alexa Fluor 555-conjugated streptavidin (S32355; Thermo Fisher Scientific Inc.) in TBS for 2 hours.

DRG sections hybridized with the cRNA probe were incubated overnight with

1:1000-diluted alkaline phosphatase-conjugated sheep anti-DIG antibody (11093274910; Sigma-Aldrich) and 1:500-diluted rat anti-HA antibody. After washing, the sections were incubated with 8 µg/ml Alexa Fluor 488-conjugated goat anti-rat IgG antibody for 1 hour and then reacted with an HNPP Fluorescence Detection kit (HNPP/FastRed, 11758888001; Sigma-Aldrich) for 30 minutes.

3.9 Image acquisition and cell counting

Sections were mounted on glass slides and coverslipped. Whole section images were obtained with an inverted fluorescent microscope (BZ-X; Keyence Co., Osaka, Japan) with a 10× objective lens. Unstitched confocal images were acquired under a confocal microscope (FV1200; Olympus Co., Tokyo, Japan) with 10×, 20×, and 40× objective lenses. To count mTFP1-positive cells in striatal compartments, stitched confocal images covering the striatum in the sections were acquired using a confocal microscope (LSM 700; Carl Zeiss, Jena, Germany) with a 20× objective lens. Cytoarchitectonic areas were determined on the images with NeuN immunoreactivity according to the atlas (Paxinos & Franklin, 2012). Epifluorescence images of whole embryos at E14.5 were acquired with a stereomicroscope (M205C; Leica Microsystems GmbH, Wetzlar, Germany). To measure the percentage of the HA-mTFP1-ir cells that showed *Oprm1* mRNA signals, at least two different confocal images were acquired with a 40× objective lens in each area presented in Figure 3. The data were averaged across three mice.

3.10 Quantitative analysis of mTFP1-positive cells in the striatal compartments

Both hemispheres from three MOR-CreER^{KI/KI}; R26-CAG-LoxP-mTFP1 mice treated with Tm were used for analysis (n = 6). For each mouse, a coronal section matched to rostral (bregma +1.21 mm ± 0.12 mm), middle (+0.49 mm ± 0.12 mm), and caudal (−0.23 mm ± 0.12 mm) striatum were used. Striosomes were identified on the confocal images of MOR IHC as characteristic patchy areas that showed higher MOR immunoreactivity than the surrounding regions in the striatum. Boundaries between the striosome and matrix were manually outlined on

the images using ImageJ (NIH, Bethesda, MD, USA). These outlines were overlaid on the mTFP1 fluorescence images, and then all of the mTFP1-positive cells in the striatum were classified as striosomal or matrix cells. Areas of striatum were defined using the atlas (Paxinos & Franklin, 2012). The cell density (number of mTFP1-positive cells per mm²) was determined by dividing the total number of mTFP1-positive cells in the striosomes or matrix by the total area of striosomes or matrix, respectively. Statistical comparisons were performed with a two-tailed Student's *t* test with Bonferroni's correction, and the difference was considered as significant when the *p* value was < 0.05.

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Figure legends

Figure 1 Generation of the MOR-CreER mouse line. **(a)** A schematic illustration of the targeting strategy. The translational stop site (Stop) in exon 4 of the *Oprm1* gene was replaced by the T2A sequence, iCreER^{T2}, and the FRT-flanked Neo cassette as indicated by gray boxes. Black boxes indicate the protein-coding region in exon 3 and 4 of the *Oprm1* gene. Triangles indicate FRT sites. The Diphtheria toxin A cassette (DTA) was used for negative selection. Arrows indicate positions and directions of PCR primers. **(b)** Genotyping of the targeted ES cell clones. Homologous recombination of 5' and 3' ends was confirmed by PCR using primers F1 and R1 (2567 bp) and primers F2 and R2 (8142 bp), respectively. **(c)** Detection of the Neo cassette excision. The excision of the FRT-flanked Neo cassette was confirmed by absence of the PCR product using primers F3 and R3. **(d)** Genotyping of the MOR-CreER line. The wildtype and knock-in alleles were confirmed by PCR using F4 and R4 primers (WT, 448 bp) and F4 and R5 primers (KI, 754 bp), respectively. **(e, f)** Tamoxifen (Tm)-dependent induction of recombination in MOR-CreER mice. A strong mTFP1 reporter signal enhanced by Alexa Fluor 488-conjugated secondary antibody was observed in Tm-treated MOR-CreER^{KI/KI}; R26-CAG-LoxP-mTFP1 mice; by contrast, there was minimal background fluorescence without Tm-treatment. **(e', f')** NeuN immunoreactivity was visualized with Alexa Fluor 555 for counterstaining. Scale bar, 2 mm.

Figure 2 Inducible Cre activity in the adult nervous system. **(a–m)** Representative coronal sections obtained from a single MOR-CreER^{KI/KI}; R26-CAG-LoxP-mTFP1 mouse treated with three rounds of 5-day Tm administration are presented. The fluorescence signal of mTFP1 was enhanced by Alexa Fluor 488. AHiPM, amygdalohippocampal area, posteromedial part; Amb, ambiguous nucleus; AOE, anterior olfactory area external part; Aul, primary auditory cortex; BST, bed nucleus of stria terminalis; Ce, central amygdaloid nucleus; CL, centrolateral thalamic nucleus; CM, central medial thalamic nucleus; DC, dorsal cochlear nucleus; DEn, dorsal endopiriform nucleus; DH, dorsal horn; DLEnt, dorsolateral entorhinal cortex; DRC, dorsal raphe nucleus, caudal part; DRG, dorsal root ganglion; Gl, glomerular layer of the olfactory bulb; GrO, granule cell layer of the olfactory bulb; I, intercalated nucleus of amygdala; IC, inferior colliculus; IPN, interpeduncular nucleus; IRT, intermediate reticular nucleus; LH, lateral hypothalamic area; LPBE, lateral parabrachial nucleus, external part; M1, primary motor cortex; Me, medial amygdaloid nucleus; MHb, medial habenula; MM, medial mammillary nucleus, medial part; MPA, medial preoptic area; MPO, medial preoptic nucleus; OFC, orbitofrontal cortex; PAG, periaqueductal gray; PC, paracentral thalamic nucleus; PV, paraventricular thalamic nucleus; Rh, rhomboid thalamic nucleus; RMg, raphe magnus; RSG, retrosplenial gyrus; S1, primary somatosensory cortex; SC, superior colliculus; Sol, solitary nucleus; Sp5, spinal trigeminal nucleus; Str, striatum; V1, primary visual cortex; Xi, xiphoid thalamic nucleus. Scale bar, 1 mm in **(m)** applies to all of the panels.

Figure 3 Cre-mediated recombination efficiency in MOR-CreER^{KI/KI}; R26-CAG-LoxP-mTFP1 adult mice. **(a–h)** HA-mTFP1 immunoreactivity and *Oprm1* mRNA in the sections of Tm-treated mice were visualized by Alexa Fluor 488 (green) and Alexa Fluor 555 or FastRed (magenta), respectively. *Oprm1* mRNA signals were observed in almost all of the HA-mTFP1-immunoreactive cells. The same abbreviations as in Figure 2 are used. Scale bar, 50 µm in **(h'')** applies to all of the panels.

Figure 4 Striosome-predominant recombination in the adult striatum. **(a–a’)** A representative coronal section at the level of the rostral striatum obtained from the Tm-treated MOR-CreER^{KI/KI}; R26-CAG-LoxP-mTFP1 mice is presented. Reporter mTFP1 fluorescence (green) was predominantly distributed in the striosomes that were also visualized by mu opioid receptor (MOR) immunoreactivity (Alexa Fluor 555, magenta). **(b–b’)** High magnification confocal images in the regions indicated by the white boxes in the upper panels. **(c)** Quantitative analysis of cell density of mTFP1-positive cells in striosomes and matrix. Average cell density was determined in the rostral (bregma +1.21 mm ± 0.12 mm), middle (+0.49 mm ± 0.12 mm), and caudal (–0.23 mm ± 0.12 mm) coronal sections (n = 6 hemispheres). The density of mTFP1-positive cells in the striosomes is significantly higher than in the matrix. Columns and error bars represent the mean ± standard deviation, respectively. *** indicates $p < 0.0001$ with Bonferroni's correction. Scale bars in **(a’)** and **(b’)** represent 500 and 50 µm, respectively.

Figure 5 Spatiotemporal control of transgene expression in MOR-positive cells in the adult striatum. **(a)** Time course of adeno-associated virus (AAV) vector injection (AAV9-hSyn-DIO-EGFP), tamoxifen (Tm) administration, and perfusion fixation. **(b)** A representative image of EGFP fluorescence in the AAV-injected striatum (Str) obtained from Tm-treated MOR-CreER^{KI/WT} animals. **(c–c’)** Transgene expression (EGFP, green) recapitulated the shape and distribution of striosomes visualized by MOR-immunoreactivity (Alexa Fluor 555, magenta). **(d–d’)** High magnification images in the regions indicated by the white boxes in the upper panels. Scale bars, 1 mm in **(b)**, 200 µm in **(c’)**, and 50 µm in **(d’)**.

Figure 6 Temporally controlled recombination in the developing striatum of MOR-CreER^{KI/KI}; R26-CAG-LoxP-mTFP1 mice. **(a)** The reporter fluorescence of mTFP1 enhanced by immunolabelling (Alexa Fluor 488, green) was observed in the striatum (Str) at embryonic day 12.5 (E12.5), following Tm administration at E11.5. DAPI (blue) was used for the counterstain. **(b)** A whole embryo (E14.5) treated with Tm at E12.5. Intense mTFP1 fluorescence was

detected in the forebrain corresponding to the striatum (arrow). **(c–c’)** Double fluorescence imaging for mTFP1 enhanced by immunolabelling (Alexa Fluor 488, green) and dopamine and cAMP-regulated phosphoprotein (DARPP32) immunoreactivity (Alexa Fluor 555, magenta) in a postnatal day 0 (P0) newborn mouse following E17.5 Tm treatment. Note that mTFP1-expressing cells were more diffusely distributed in the striatum than DARPP32-immunoreactive cells. **(d)** A high magnification image in the region indicated by the white box in **(c’)**. LGE, lateral ganglionic eminence; LV, lateral ventricle. Scale bars, 200 μ m in **(a, c’)** and 20 μ m in **(d)**.

Figure S1 Inducible Cre activity in the adult spinal cord and DRG. Confocal images of spinal dorsal horn **(a)** and DRG **(b)** obtained from a Tm-treated MOR-CreER^{KI/KI}, R26-CAG-LoxP-mTFP1 mouse are presented. The fluorescence signal of mTFP1 was enhanced by Alexa Fluor 488. Scale bar, 50 μ m in **(b)**.

Figure S2 Double labeling for HA-mTFP1 immunoreactivity and *Oprm1* mRNA in the layer V of the somatosensory cortex (S1) of a Tm-treated MOR-CreER^{KI/KI}, R26-CAG-LoxP-mTFP1 adult mouse. HA-mTFP1 immunoreactivity **(a, b)** was visualized with Alexa Fluor 488 (green) and hybridization signals with an antisense cRNA probe **(a’)** or a sense cRNA probe **(b’)** with Alexa Fluor 555 (magenta). The sense probe detected no signal above the background. Scale bar, 50 μ m in **(b’)** applies to all of the panels.

Figure S3 Double labeling for HA-mTFP1 immunoreactivity and the iCreER^{T2} mRNA in the brain of a Tm-treated MOR-CreER^{KI/KI}, R26-CAG-LoxP-mTFP1 adult mouse. **(a–e)** HA-mTFP1 immunoreactivity and iCreER^{T2} mRNA signals were visualized with Alexa Fluor 488 (green) and Alexa Fluor 555 (magenta), respectively. The same abbreviations as in Figure 2 are used. Scale bar, 50 μ m in **(e’)** applies to all of the panels.

Figure 1

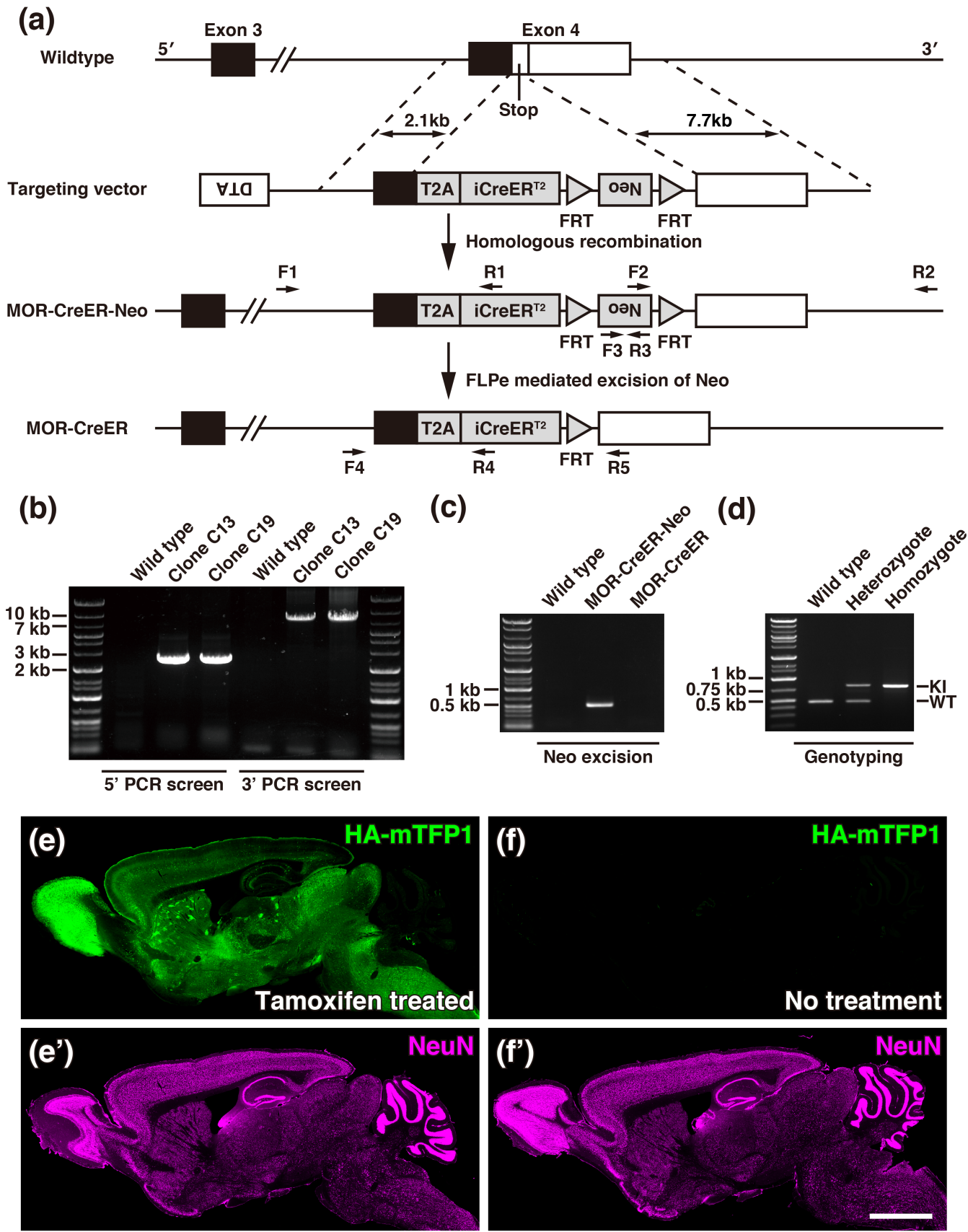


Figure 2

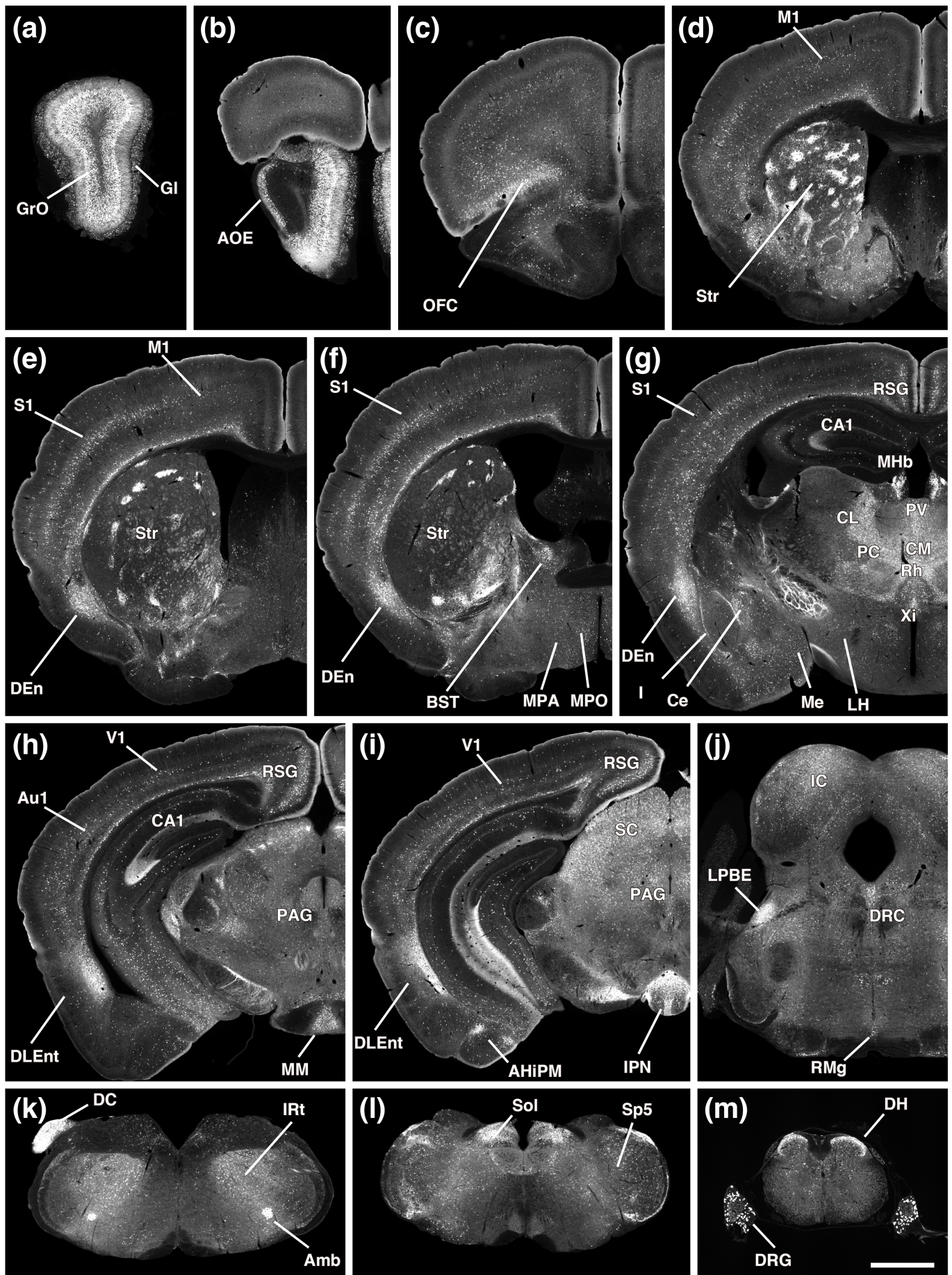


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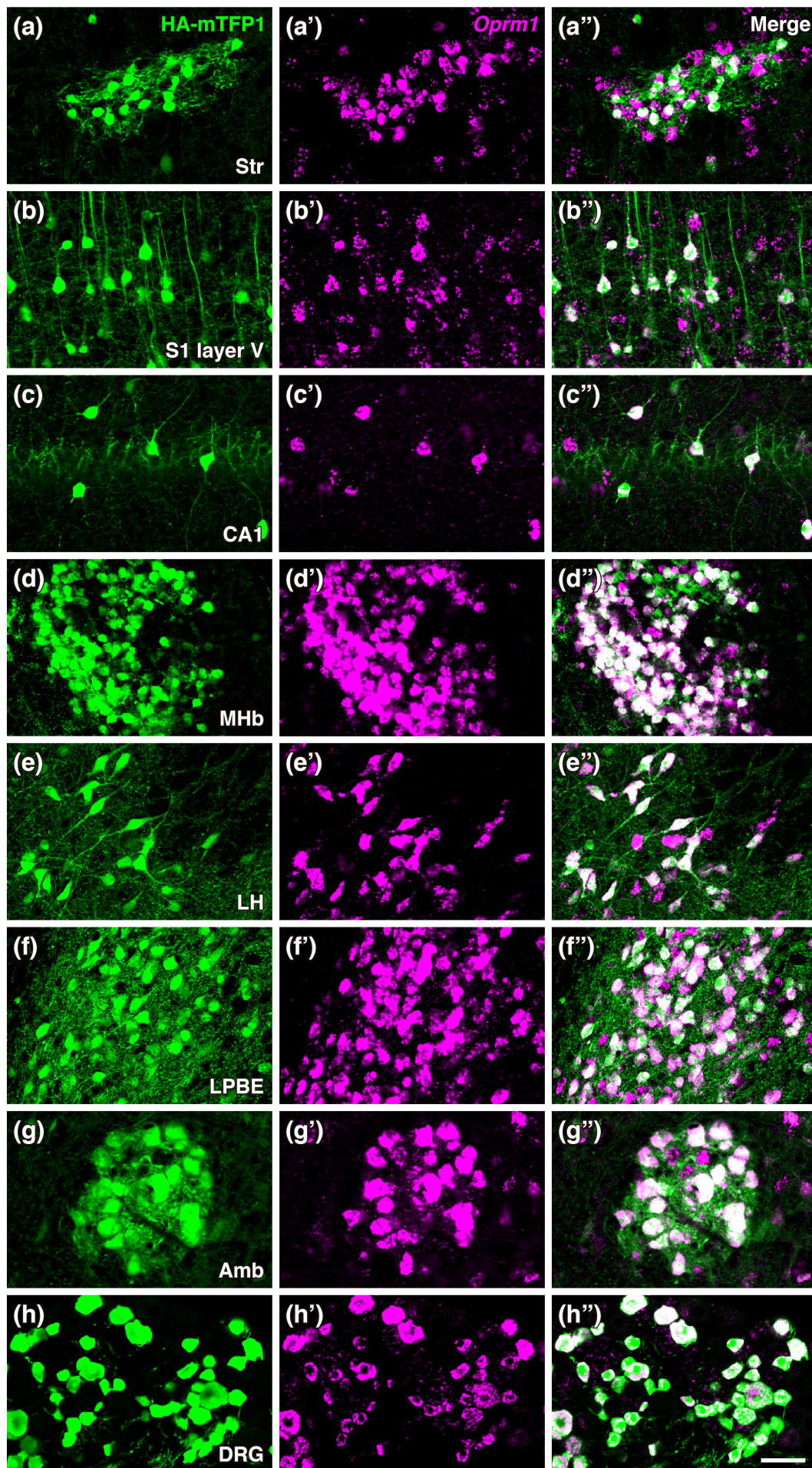


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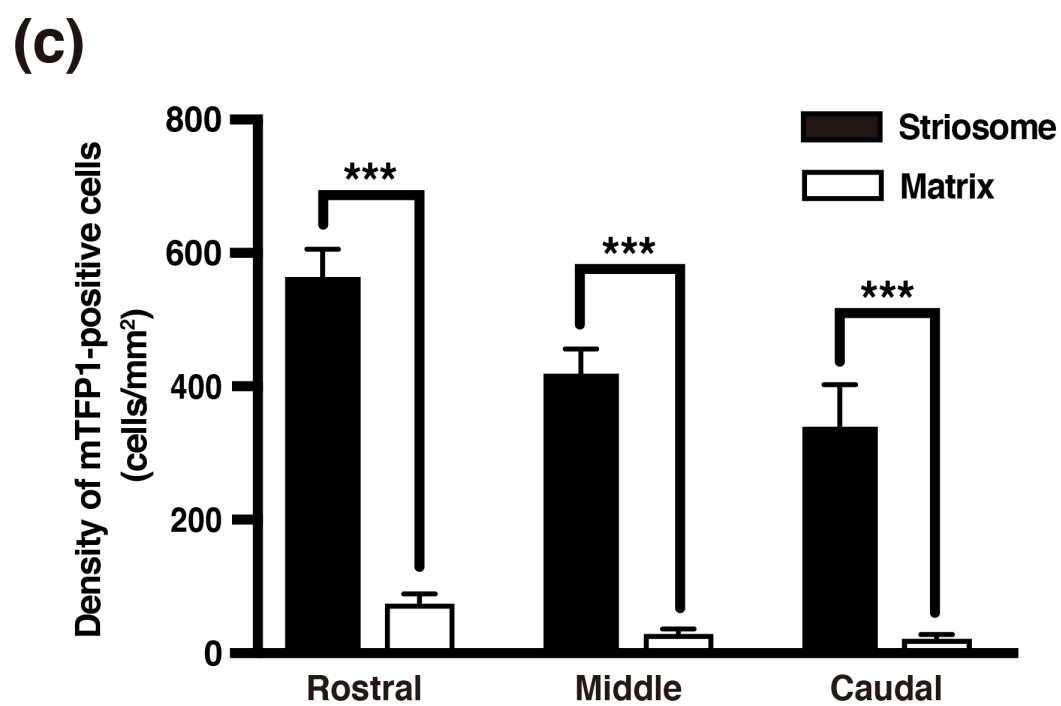
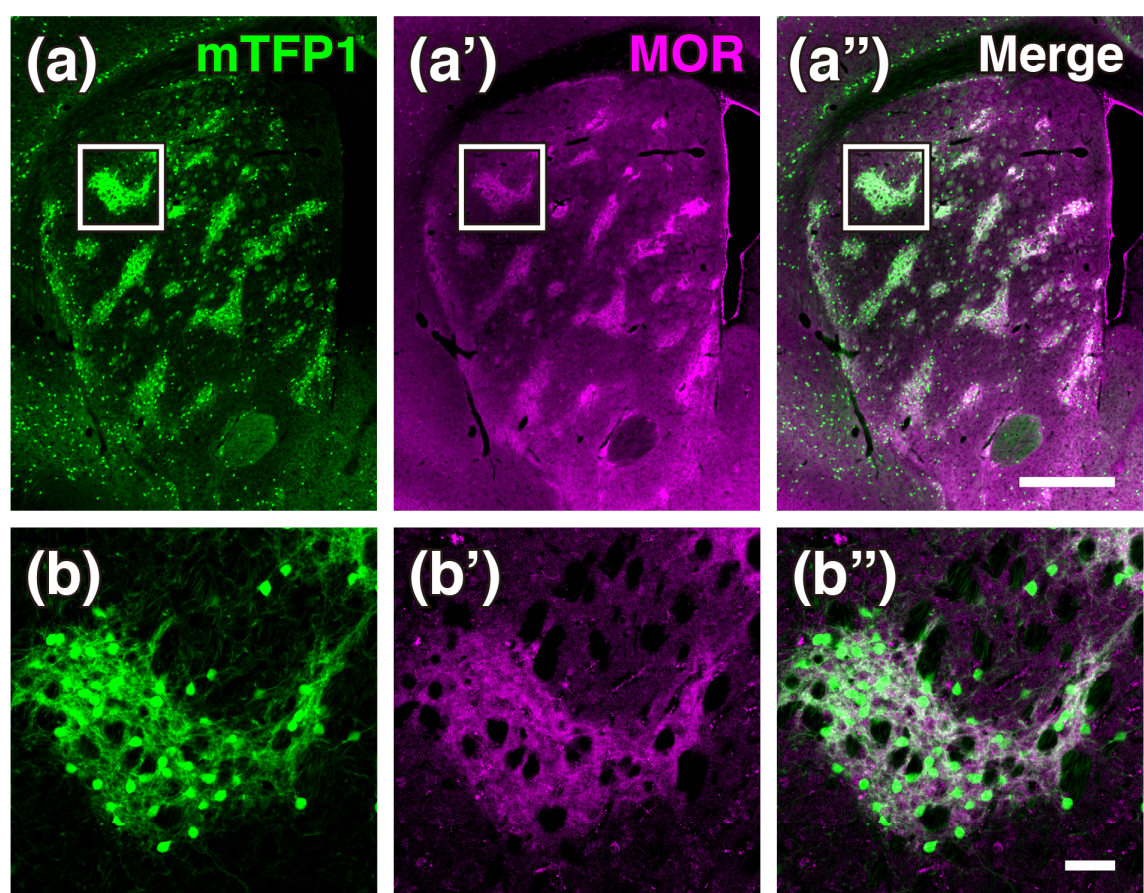


Figure 5

(a)

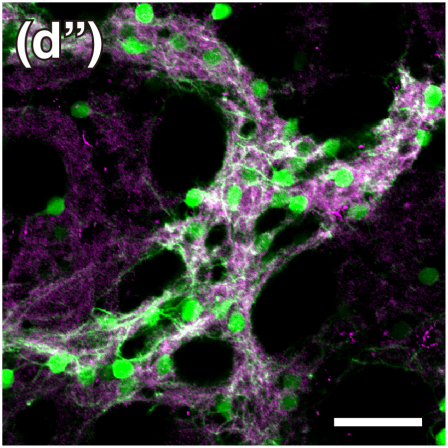
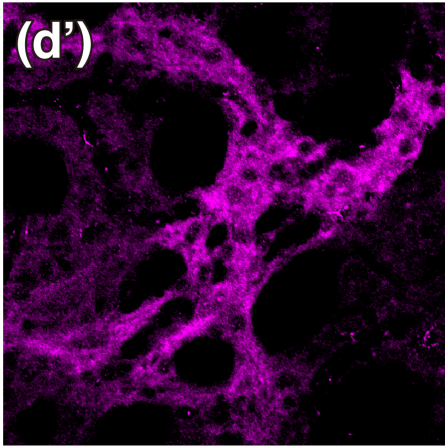
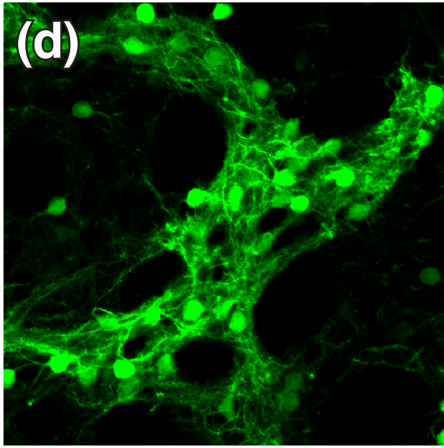
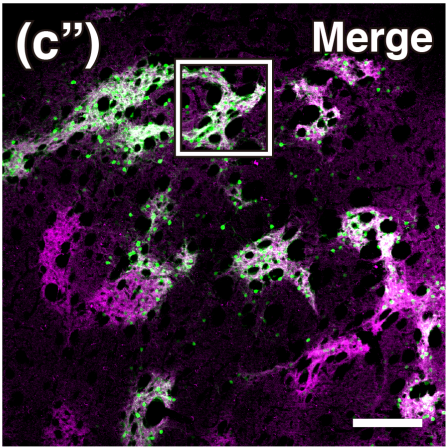
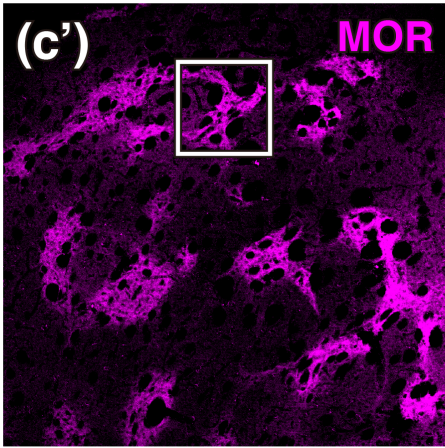
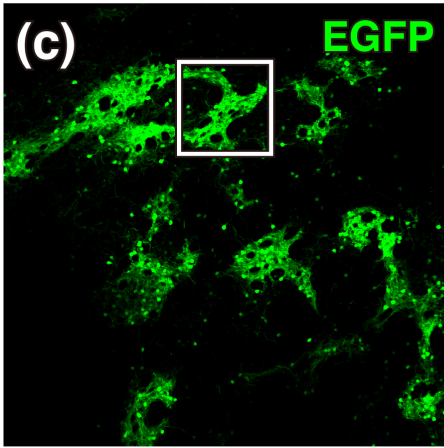
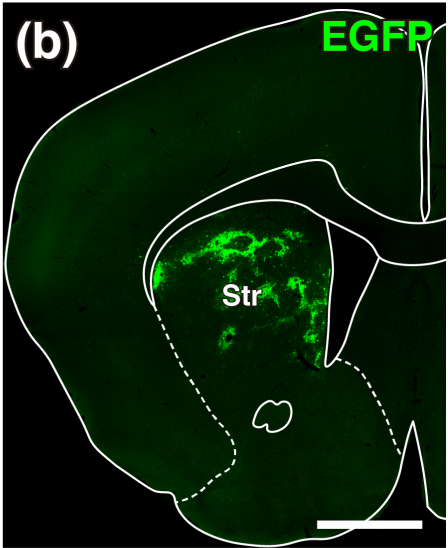
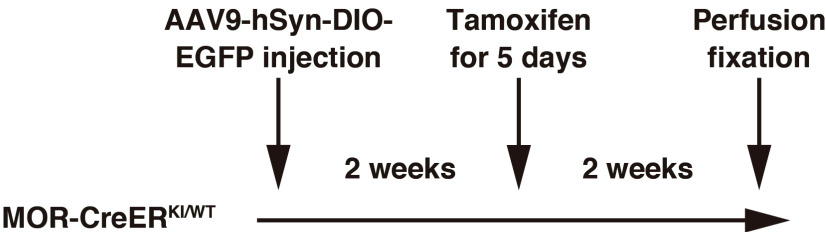


Figure 6

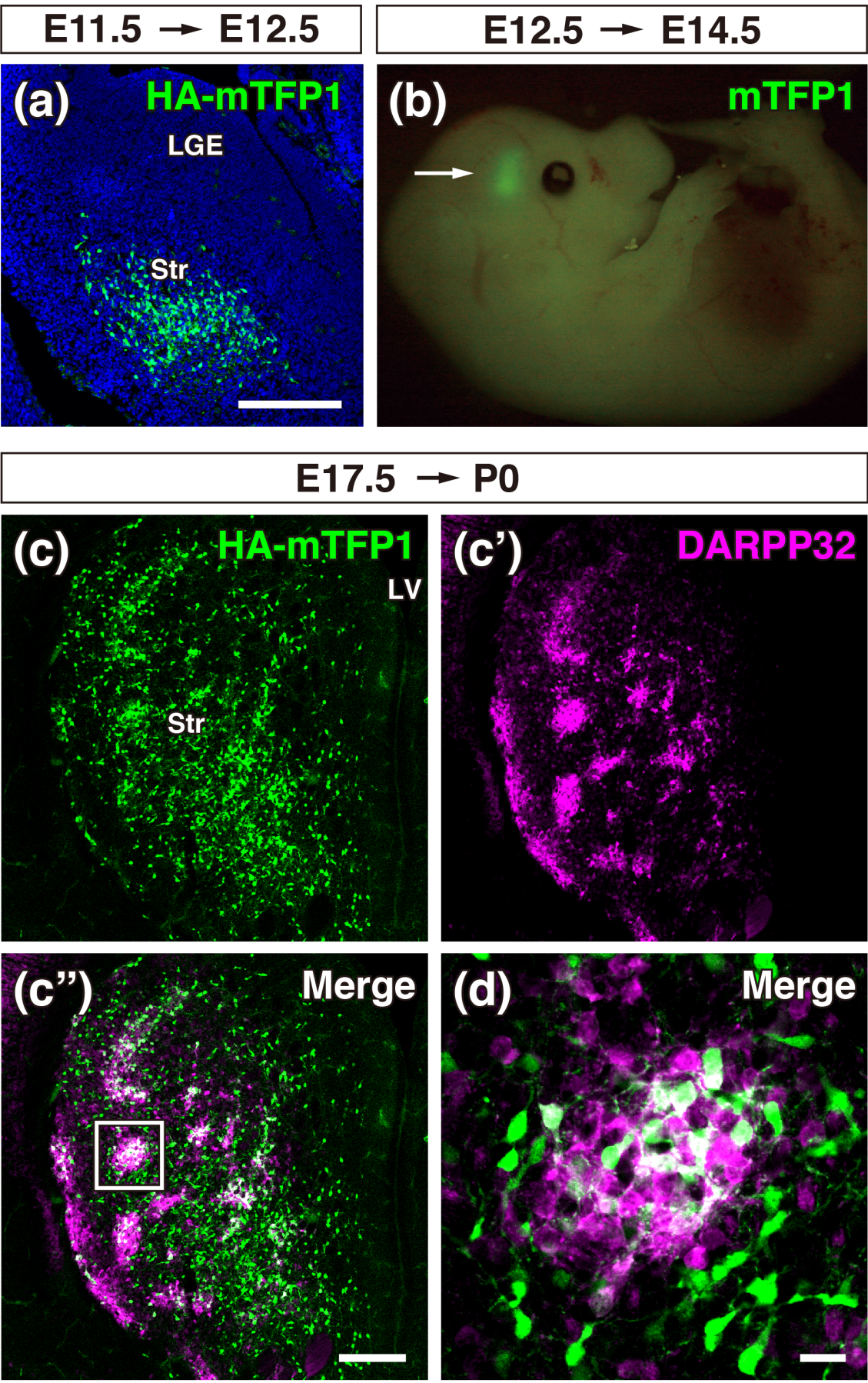


Figure S1

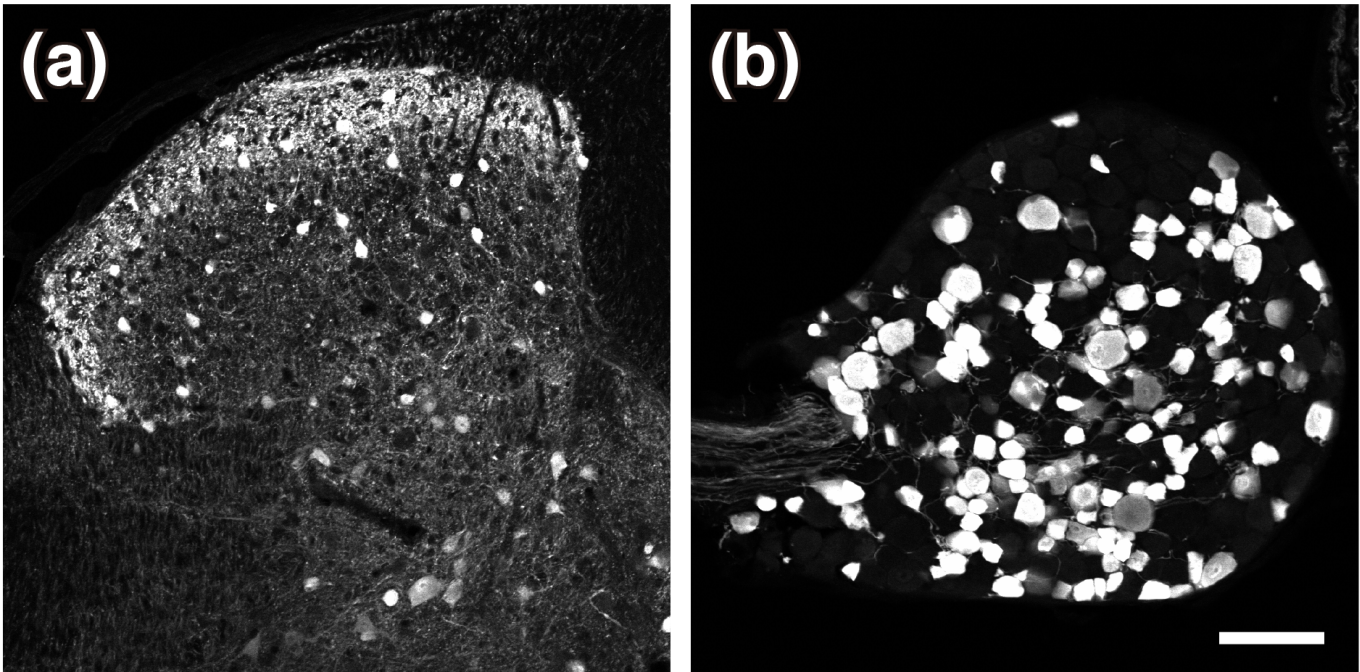


Figure S2

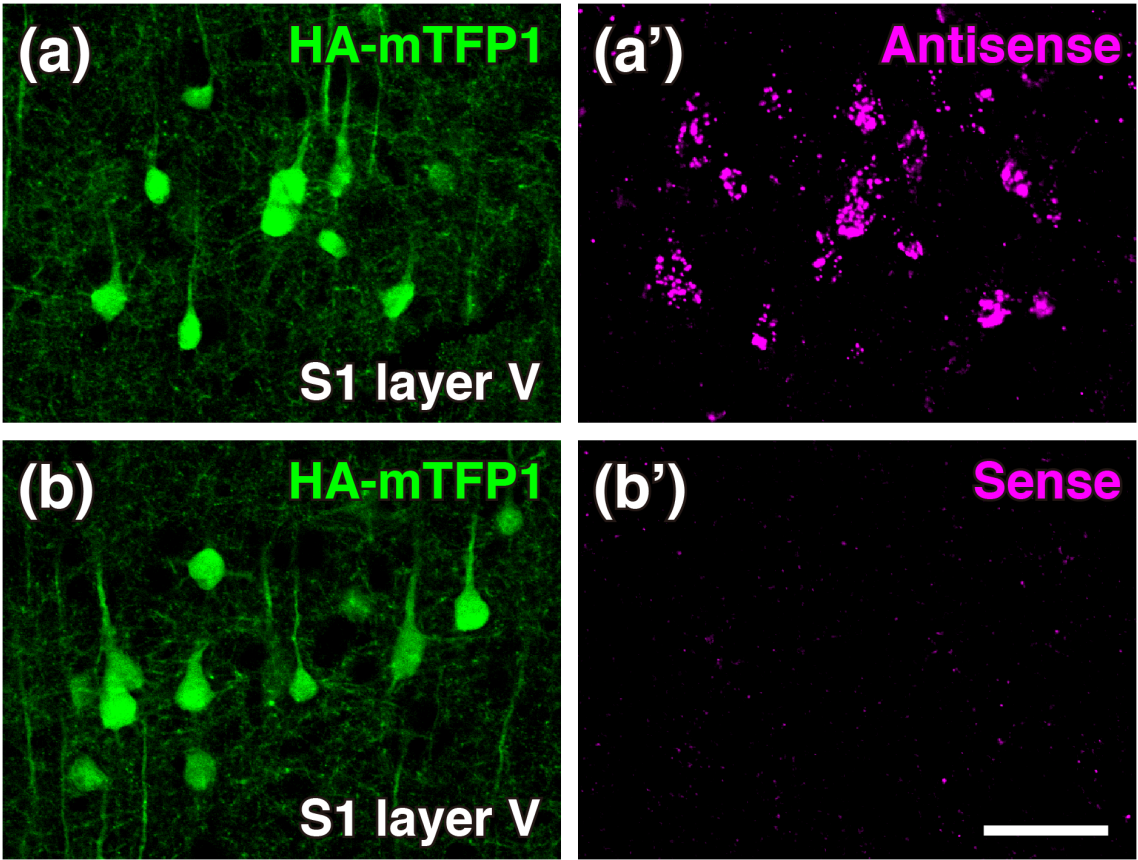


Figure S3

