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# Identification of neuron-type specific promoters in monkey genome and their functional validation in mice

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Running title: Neuron-type specific promoters from monkey

**Abstract**

Viral gene delivery is one of the most versatile experimental techniques for elucidating the mechanisms underlying brain dysfunction, such as mental and neurodegenerative disorders. Due to the complexity of the brain, expression of genetic tools, such as channelrhodopsin and calcium sensors, often has to be restricted to a specified cell type within a circuit implicated in these disorders. Only a handful of promoters targeting neuronal subtypes are currently used for viral gene delivery. Many of them use human genomic elements although used typically in mice or rats. Here, we isolated conserved promoter regions of several subtype-specific genes from the macaque genome and investigated their functionality in the mouse brain when used within lentiviral vectors (LVVs). Immunohistochemical analysis revealed that transgene expression induced by the promoter sequences for somatostatin (SST), cholecystokinin (CCK), parvalbumin (PV), serotonin transporter (SERT), vesicular acetylcholine transporter (vAChT), substance P (SP) and proenkephalin (PENK) was largely colocalized with specific marker for the targeted neuronal populations. Moreover, by combining these results with in silico predictions of transcription factor binding to the isolated sequences, we identified transcription factors possibly underlying cell-type specificity. These findings lay a foundation for the expansion of the current toolbox of promoters suitable for elucidating these neuronal phenotypes.

## Introduction

Abnormal neuronal activity causes the symptoms of a variety of neuropsychiatric disorders, including Alzheimer's disease, Parkinson's disease, autism, schizophrenia, and major depression[1-5]. Genetic tools such as optogenetic or chemogenetic actuators and sensors for intracellular molecules[6-10], are among the most versatile experimental techniques for elucidating the mechanisms underlying these disorders. Indeed, application of these tools in rodents has revealed the neural mechanisms contributing to these disorders [11-15]. At the same time, monkeys have ability to perform complex tasks due to their high intelligence. Moreover, the brain structures of humans and monkeys are very similar, especially the cerebral cortex, which plays a key role in memory, learning, emotion, and cognition. Some brain nuclei, such as the pulvinar nucleus, is present only in primates but not in rodents. Furthermore, genome sequence of monkeys is highly homologous to that of humans[17, 18]. Therefore, the application of neuron type-specific genetic tools to monkeys should not only help to extrapolate observations made in rodents to humans, but also provides deep insights into the mechanisms of brain function in health and disease.

It is necessary to reach sufficient level of expression of genetic tools in a specific population of neurons for manipulation and monitoring of its neural activity[19-23]. In rodents, especially in mice, this has been often achieved by utilizing Cre-driver lines and Cre-dependent adeno-associated viral vectors[24, 25]. On the other hand, in other species where a Cre-driver line is not readily available, such as in monkeys, viral vectors with cell-type specific promoters are indispensable for achieving cell-type specific transgene

expression.

In this context, human synapsin (hSyn) and mouse calmodulin kinase II α (mCaMKIIα) promoters were used to target neurons in monkeys[26-31]. Recently, Stauffer et al. have demonstrated that a short tyrosine hydroxylase (TH) promoter can transduce dopamine neurons with high neuron-type specificity, and have optogenetically manipulated their activity[32]. El-Shamayleh et al. have shown that an L7 promoter allows specific expression in Purkinje cells in the cerebellum and is sufficiently active for optogenetic manipulation of these cells in monkeys[33]. Interestingly, both of these reports employed promoter sequences that were isolated from rodents and were effective in monkeys. Binding motifs of transcription factors, which underlie cell-type specific promoter activity, are highly conserved across species[34]. Moreover, a systematic comparison of mammalian genomes has revealed that promoter regions are evolutionarily conserved compared to intronic regions or the whole genome[35]. These results indicate that upstream regions of protein coding sequences are good candidates for development of cell-type specific promoters active across species. Indeed, we have shown that lentiviral vectors (LVVs) with a 2-kb rodent tryptophan hydroxylase 2 (TPH2) promoters, which is well conserved across species, are capable of inducing sufficient expression of optogenetic tools specific to rodent serotonergic neurons for manipulation of these neurons *in vivo*[23]. These considerations prompted us to screen the activity of evolutionarily conserved promoter sequences from monkeys in the mouse.

In this study, we isolated promoter candidates for several well-established neuronal markers[36-40], somatostatin (SST), cholecystokinin (CCK), parvalbumin (PV), serotonin

transporter (SERT), choline acetyltransferase (ChAT), substance P (SP), and enkephalin (PENK), from the genome of crab-eating macaques (*Macaca fascicularis*) through comparative analysis of upstream regions between mice and monkeys. We investigated the promoter activity of the isolated sequences in mice using LVVs[23,41] and found that several of these promoter candidates were capable of inducing transgene expression in a neuron-type specific manner.

## Materials and methods

### Animals

Adult male and female C57BL/6J mice (8-16 weeks old; Nihon SLC, Shizuoka, Japan) were used in this study. All animal experiments were performed in accordance with the ethical guidelines of the Kyoto University Animal Experimentation Committee, and were approved by the Kyoto University Animal Experimentation Committee. Mice were housed in groups (no more than 6 mice in an individual cage) with free access to food and water and kept under constant ambient temperature (24 ± 1 °C) and humidity (55 ± 10 %) and a 12-hr light-dark cycle. The sample size was similar to that in previous report[23] and was estimated to be sufficient to determine the specificity of each promoters. Mice were randomly assigned to experimental groups. Blinding was not performed.

### Isolation of the upstream sequence of neuronal markers and vector construction

Genomic DNA of crab-eating macaque (*Macaca fascicularis*) was isolated from blood by using QIAamp DNA Blood Minikit (Qiagen, Hilden, Germany). Upstream sequences of

somatostatin (SST), cholecystokinin (CCK), parvalbumin (PV), serotonin transporter (SERT), vesicular acetylcholine transporter (vAChT), substance P (SP) and enkephalin (PENK) were isolated from crab-eating macaque genomic DNA by PCR. PCR was performed with KOD FX Neo (Toyobo, Osaka, Japan) or Q5 DNA polymerase (New England Biolabs, Ipswich, MA, USA). Sequences of primers are shown in Supplementary Table 1. PCR-amplified upstream sequences were digested with *Mlu*I and *Not*I and ligated into the lentiviral plasmid backbone, pTYF-super-mTPH2-Venus-WPRE[23], which was pre-digested with *Mlu*I and *Not*I. All ligation reactions were performed with DNA Ligation Kit Mighty Mix (Takara Bio, Otsu, Japan). Transgene Venus[42] is a variant of eYFP and was used as reporter gene in this study. The structure of resulting constructs was pTYF-5xGal4-binding sequences-promoter-Venus-IRES-Gal4p65-WPRE, thus incorporating the positive enhancer feedback loop[41, 43]. The plasmids containing the isolated promoters will be deposited to Addgene.

**Lentiviral vector production**

Production and purification of LVVs were performed as described previously[23, 44]. Briefly, LVVs were produced by transient cotransfection of Lenti-X 293T cells (Clontech, Mountain View, CA, USA) with a pTYF shuttle vector[45] (15.5 µg), a packaging vector pNHP (31.2 µg), and a plasmid for envelope protein expression (vesicular stomatitis virus glycoprotein, VSVG, 12.4 µg). After 16-18 hrs of incubation, the supernatant was harvested, and fresh media was added to the culture. After 30 hrs of incubation, the supernatant was collected and mixed with that of the first harvest. The supernatants were

filtered through a 0.45-μm pore PVDF membrane (Millex-HV, Merck Millipore, Billerica, MA, USA) and ultracentrifuged for 2 hr 40 min at 20,000 rpm in an SW-28 rotor (Beckman-Coulter, Brea, CA, USA). The resulting pellet was suspended in phosphate buffered saline (PBS) and stored at -80 °C. The titers of LVVs were measured by p24 ELISA kit (R&D systems, Minneapolis, MN, USA), and estimated to be approximately $1\times10^{10}$ IU/mL.

**Stereotaxic surgery**

Stereotaxic surgeries were conducted using a small animal stereotaxic frame (Narishige, Tokyo, Japan) and performed according to the Brain Atlas[46]. The sites of injections were selected based on the known expression pattern of each of the target genes. Mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p., Kyoritsu Seiyaku, Tokyo, Japan). Under pentobarbital anesthesia, all mice were injected with 1 μL of LVV. The following coordinates (in mm) were used for stereotactic injections: SST and CCK, cingulate cortex (AP +0.98 mm, ML 0.3 mm, DV +2.0 mm from bregma) and M2 cortex (AP +0.74 mm, ML 1.0 mm, DV +1.5 mm from bregma); PV, thalamic reticular nucleus (AP -0.70 mm, ML 1.1 mm, DV +4.2 mm from bregma); SERT, dorsal raphe nucleus (AP -4.3 mm, ML 1.2 mm, DV +3.6 mm, 20° from bregma); vAChT, lateral dorsal tegmentum (AP -5.02 mm, ML 0.5 mm, DV +3.3 mm from bregma); and SP and PENK, striatum (AP +0.38 mm, ML 2.0 mm, DV +3.5 mm from bregma).

**Immunohistochemistry**

One week after LVV injection, mice were perfused transcardially with PBS and 4% paraformaldehyde (Nacalai Tesque, Kyoto, Japan) in 0.1 M phosphate buffer (pH 7.4) under pentobarbital anesthesia. The brain was removed from the skull, and stored in 15 % sucrose in 0.01 M PBS at 4°C overnight, and 30-μm-thick frozen sections were prepared by freezing microtome (Leica CM3050S; Leica Biosystems, Nussloch, Germany) and stored at -80°C until immunohistochemical processing. For immunohistochemistry, the sections were immersed in 0.25% Triton-X 100 (Nacalai Tesque) for permeabilization and then incubated with each primary antibody under appropriate conditions. Details of the primary antibodies and immunostaining conditions are described in Supplementary Table 2. After washing with PBS, the glass slides were incubated in secondary antibody solution, specifically Alexa Fluor 488- or 594-labeled donkey anti-rabbit, anti-goat, anti-sheep and anti-rat IgG (1:200; Life Technologies, Carlsbad, CA, USA) for 2 hrs at room temperature. After washing with PBS, sections were mounted with Fluoromount Plus (Diagnostic Biosystems, Pleasanton, CA, USA). Immunoreactivity was visualized by confocal microscopy (Fluoview FV10i, Olympus, Tokyo, Japan). In some cases, antigen retrieval by citrate buffer or HistoVT One (Nacalai Tesque) was performed before permeabilization (see Supplementary Table 2).

**_In silico_ prediction and comparison of transcription factor binding**

An open-access database of transcription factor binding profiles, ConSite[47], was used for in silico prediction of transcription factor (TF) binding to the sequence. In this method, the program scans the isolated promoter sequences to examine whether a set of TF binds

to each fragment of sequences or not based on a matrix tabulating observed nucleotides in each position of the protein-DNA interface[47], and calculates the scores which are normalized to 0-100% range. For all isolated sequences, the TF score cutoff was set to 80%.

**Data Analysis**

Specificity was evaluated by the colocalization of Venus with a canonical reporter gene for each cell type. All values were expressed as mean ± standard error of mean.

**Results**

**Isolation and functional validation of promoter candidates for SST, CCK, and PV neurons**

In the cerebral cortex, there are excitatory and inhibitory neurons. Specific expression using viral vectors in excitatory and inhibitory neurons is often achieved by the CaMKIIα promoter and the Dlx promoter, respectively[48-50]. However, inhibitory neurons are further classified into several subtypes, including somatostatin (SST)-, cholecystokinin (CCK)-, and parvalbumin (PV)-positive neurons. Importantly, short promoters that are active specifically in these subtypes of inhibitory neurons have not yet been identified in any species including mice. First, we identified conserved promoter region upstream of the SST gene through homology analysis of mice and crab-eating macaque using zPicture[51] (Fig. 1A). We found that sequence just upstream of the SST start codon was highly

conserved among these species. We produced LVVs bearing this conserved region upstream of Venus (LVV-SST-0.3 kb::Venus). One week after injection of LVV-SST-0.3 kb::Venus into the cingulate cortex of mice, the specificity of the promoter candidate was evaluated by immunohistochemical analysis. We found that 93.8 ± 4.1 % of Venus-immunoreactive cells were also SST-immunoreactive (n = 4 mice; Fig.1B, C). This result indicates that this conserved region is active specifically in SST-positive neurons. Similarly, we isolated promoter candidates containing conserved promoter regions upstream of the CCK gene (Fig. 2A). LVVs containing these promoters (LVV-CCK-0.5 kb::Venus and LVV-CCK-3.9 kb::Venus) were injected into the cingulate cortex of mice. One week after viral injection, the specificity of the promoters was analyzed immunohistochemically. A large proportion of GFP expression was confined to CCK-immunoreactive cells in animals injected with LVV-CCK-0.5 kb::Venus (colocalization rate 88.0 ± 3.3 %, n = 3 mice; Fig. 2B, C), whereas GFP expression was observed not only in CCK-immunonegative cells but also other cells in animals injected with LVV-CCK-3.9 kb::Venus (colocalization rate 50.9 ± 3.3 %, n = 3 mice; Fig. 2D, E). These results suggest that CCK neuron-specific promoter activity is coded in the proximal region of the upstream region of the CCK gene and addition of a more distal region leads to nonspecific transgene expression. Further, we isolated a conserved promoter region upstream of the PV gene. LVVs bearing two promoter candidates (LVV-PV-0.8 kb::Venus and LVV-PV-1.8 kb::Venus) were injected into the reticular nucleus, because PV-positive neurons are more densely distributed in the reticular nucleus than in the cerebral cortex. Immunohistochemical analysis revealed that 84.0 ± 1.4 % of GFP-immunoreactive cells

also expressed PV in animals injected with LVV-PV-0.8 kb::Venus (n = 3 mice; Fig. 3B, C), whereas 79.3 ± 0.5 % of GFP-immunoreactive cells were immunopositive for PV in animals injected with LVV-PV-1.8 kb::Venus (n = 3 mice; Fig. 3D, E). These results indicate that as little as 0.8 kb upstream region of the PV gene is sufficient for preferential expression in this neuronal subtype.

We investigated whether the isolated promoters induce strong transgene expression so that fluorescence of transgene Venus is detectable without immunohistochemical enhancement. We found that strong Venus fluorescence was induced by LVV-SST-0.3kb::Venus, LVV-CCK-0.5kb::Venus, or LVV-PV-0.8kb::Venus (Supplementary Fig. S1A-C).

**Isolation and functional validation of promoter candidates for serotonergic and cholinergic neurons**

Serotonin and acetylcholine transmitter systems are critical for a variety of brain functions such as mood regulation, learning, reinforcement of behavior, and nociception[23, 52, 53]. We previously reported that the proximal promoter upstream of the mouse and rat TPH2 gene were specifically active in serotonergic neurons in mice and rats[23, 41, 54]. However, no selective promoter for primate serotonergic and cholinergic neurons has been reported. We identified conserved promoter region of the upstream of the serotonin transporter (SERT) gene (Fig. 4A). Two LVVs containing conserved promoter regions (LVV-SERT-0.5 kb::Venus and LVV-SERT-1.9 kb::Venus) were constructed and injected into the dorsal raphe nucleus, the largest serotonergic nucleus. Immunohistochemical

analysis revealed specific GFP expression in animals injected with the longer promoter (colocalization rate 93.8 ± 0.9 %, n = 3 mice; Fig. 4D, E), whereas the shorter was non-selective (colocalization rate 56.3 ± 1.4 %, n = 3 mice; Fig. 4B, C). Similarly, we identified conserved promoter region upstream of the vesicular acetylcholine transporter (vAChT) gene, which is a marker of cholinergic neurons[55]. Two LVVs containing conserved promoter regions (LVV-vAChT-1.1 kb::Venus and LVV-vAChT-1.8 kb::Venus) were tested in the latero-dorsal tegmental nucleus (LDTg). Similar to the result with the SERT promoters, the shorter promoter was less specific (colocalization with choline acetyl transferase, ChAT, at rate 52.3 ± 4.8 % n = 3 mice; Fig. 5B, C), while the longer promoter induced more specific GFP expression (colocalization rate 83.1 ± 3.9 % n = 3 mice; Fig. 5D, E).

Moreover, we determined whether these promoters are sufficiently strong for inducing detectable level of fluorescence of Venus. We found that Venus fluorescence induced by LVV-SERT-1.9kb::Venus was not detectable without immunohistochemical enhancement (Supplementary Fig. S1D), whereas that induced by LVV-vAChT-1.8kb::Venus was barely detectable (Supplementary Fig. S1E).

**Isolation and functional validation of promoter candidates for striatal medium spiny neurons**

The striatum is anatomically a part of the basal ganglia and plays a key role in motor function as well as decision making[56-58]. A large population of striatal neurons are medium spiny neurons (MSNs), which project into the endopeduncular nucleus (internal globus

pallidus), external globus pallidus, and substantia nigra pars reticulata[56]. There are two distinct clusters of MSNs; one expresses dopamine D1 receptors and substance P (SP) (D1-MSNs), and another expresses dopamine D2 receptors, adenosine A2A receptors and enkephalin (D2-MSNs). Previous reports have demonstrated that mouse promoters for SP and preproenkephalin (PENK) are specifically active in mouse D1-MSN and D2-MSN, respectively[59].Although previous reports used relatively long sequences containing distal upstream region and part of coding region, we used conserved promoter region upstream of the SP and PENK genes which did not contain any coding region for minimizing the promoter length (Fig. 6A, 7A). Two LVVs containing conserved promoter regions upstream of the SP gene and more distal non-conserved region for comparison were created and injected to the mouse striatum. Immunohistochemical analysis revealed that both the longer and shorter promoters induced specific GFP expression in SP-positive neurons (LVV-SP-0.8 kb::Venus; colocalization rate 91.7 ± 3.8 %, n = 3 mice, Fig. 6B, C, LVV-SP-1.7 kb::Venus; colocalization rate 87.4 ± 4.9 % n = 3 mice, Fig. 6D, E). Similarly, we designed two LVVs containing conserved promoter regions upstream of the PENK gene and injected them into the mouse striatum. We found that the longer promoter (LVV-PENK-2.2 kb::Venus) showed low specificity (colocalization rate 61.0 ± 5.8 %, n = 3 mice; Fig.7D, E), whereas the shorter promoter (LVV-PENK-0.9 kb::Venus) induced more specific GFP expression in PENK-immunoreactive neurons (colocalization rate 88.0 ± 1.7 %, n = 3 mice; Fig. 7B, C).

Next, we examined whether these promoters induce strong transgene expression so that fluorescence of transgene Venus is detectable without immunohistochemical

enhancement. We found that strong Venus fluorescence was induced by LVV-SP-0.8kb::Venus or LVV-PENK-0.9kb::Venus (Supplementary Fig. S1F, G).

**In silico prediction and comparison of transcription factor binding to identified promoters**

Sequence-specific transcription factors (TFs) play an important role in regulating the expression of target genes by binding to transcriptional regulatory regions, such as promoters and enhancers[60]. Thus, we hypothesized that different levels of promoter specificity could be due to different degrees of TF binding to the promoters. To address this issue, we utilized ConSite, an in silico prediction method for TF binding to promoter sequences[47]. We analyzed the longer promoters for each target gene by ConSite and identified TF binding sites in these promoters. Then, we counted the occurrence of each TF in the whole sequences of the longer promoters, in the sequences specific to the longer promoters, and in the sequences common to both the longer and shorter promoters (Supplementary Table 3). We found a number of TFs bound only to the sequences specific to the longer promoters or to the sequences common to both the longer and shorter promoters. We specifically focused on the differences between long and short versions of CCK and SERT promoters. In case of CCK, the shorter version was much more specific than the longer one (Fig. 2). This suggested that TFs, which bind to the sequence common to both the longer and shorter promoters, may contribute to the specificity. We found that Broad-complex 1, HNF-1, MEF2, and Suppressor of Hairless (SU(h)) were predicted to bind to the sequences common to both the longer and shorter CCK promoters but not to

the sequences specific to the longer promoters (Supplementary Table 3). Interestingly, according to the *in situ* hybridization data available from the Allen Mouse Brain Atlas[61], the Mef2c expression pattern in the cerebral cortex is very similar to the expression pattern of CCK but not to those of PV and SST (Supplementary Fig. S2), highlighting the possible involvement of this TF.

## Discussion

Promoters which are able to specifically express transgenes in sub-populations of central neurons are highly valuable tools but still are in short supply. Here we searched for promoters which contain evolutionally conserved sequences suitable for targeting an array of important neuronal phenotypes, which are common between monkey and mouse. Our assumption is that the most important regulatory elements such as binding sites of critical transcriptional factors should be retained in both species. We successfully identified several macaque promoters which specifically drove gene expression in the homologous populations of neurons in the mouse brain. However, we cannot rule out the possibility that sequences other than conserved regions in the promoter may play a key role in the specificity of the promoter. Thus, further investigation using mutated conserved sequences or randomly selected sequences from the respective promoters is needed to clearly show the importance of conserved sequences. Moreover, by using in silico TF binding prediction, we found TFs which are likely to be important for the specificity of the identified promoters. Although our initial screening for obvious reason had to be performed in the mouse, there are good reasons to expect that their selectivity

will be preserved in other species such as macaque.

Recent advances in genetic tools including optogenetic, chemogenetic, and imaging constructs have revolutionized the ability to manipulate and record neuronal activity as well as analysis of synapse-level connectivity underlying brain function[6-15, 62, 63]. However, practical application of these tools often requires sufficiently high and specific expression of these tools in defined populations of neurons. In this study, we showed that upstream regions of SST, CCK, PV, SERT, vAChT, SP, and PENK genes in crab-eating macaque are capable of directing specific expression in the relevant populations of murine neurons. Although the identified promoters except for SERT and vAChT were sufficiently strong for inducing detectable level of transgene Venus fluorescence, whether expression of various transgenes driven by newly identified promoters will be sufficiently high for manipulation and recording of neuronal activity *in vivo* remains to be seen. However, the utilization of Cre in combination with a Cre-dependent expression cassette[12, 13, 64] can dramatically enhance the expression level with preserving high cell-type specificity. Therefore, it should be possible to combine cell-specific expression of Cre with Cre-dependent viral vectors to achieve the level of expression required for cell-type specific manipulation and recording of neuronal activity even in the monkey. Indeed, Stauffer et al.[32] have successfully transduced and manipulated monkey dopamine neurons using this approach. In this study, we injected each LVV to the brain area where target neurons predominantly exist. Therefore, we cannot fully rule out the possibility that this population bias might lead to overestimation of the specificity of promoters.

Systematic analysis of promoter sequences also contributes to development of the

transgenic animals, which are widely used to gain insights into molecular mechanisms and potential therapies for a variety of diseases[65]. Transgenic macaques were first reported in 2001[66], and later in 2008[67] and 2016[68]. In contrast to the methods usually used in mice, these reports used retroviral or lentiviral vectors for transgene expression. Therefore, it is possible that the LVVs with the identified promoters may be also effective in establishing cell-type specific transgenic macaque. It is worth noting that also the transgenic common marmoset, another non-human primate, with germline transmission has been established by LVVs[69]. Considering that marmosets reach sexual maturity at 12-18 months, use of macaque promoters identified in this study or those of marmoset in generation of transgenic animals will provide new insights into a variety of mental and neurodegenerative diseases in primate models which are the best available approximation of human pathology.

While in this study we used LVV for speedy gene expression, adeno-associated virus (AAV) is currently the most popular vector used in rodent studies[70-72]. Similar to LVV, AAV provides stable and long-term gene expression in the targeted cells[73]. However, standard AAV cannot package more than 5 kb, while LVV can accommodate up to 9 kb[73, 74]. From this perspective, all of the identified promoters in this study were shorter than 2 kb which could be incorporated into AAV while leaving sufficient space for the transgenes. However, considering the episomal and concatemeric nature of AAV genomes[75], which might affect the specificity and/or expression level, their performance in the AAV backbones requires further investigation.

In conclusion, we have successfully identified promoter sequences in the macaque

genome which may act as cell specific promoters in an array of neuronal sub-types and tested them in the mouse. We believe that these promoters will be useful for further application of genetic tools in non-human primates in a cell-type specific manner.

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**Conflict of Interest**

None

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**Figure legends**

**Figure 1. Functional validation of macaque SST promoters in mouse.**

**A** (top) Sequence similarity of upstream regions between mouse and macaque. (bottom) Schematic representation of isolated promoters. **B** One week after injection of LVV-SST-0.3 kb::Venus, transgene expression was analyzed immunohistochemically. Scale bars = 200 μm (low magnification), 20 μm (high magnification). **C** Colocalization rate of SST and Venus. n = 4 mice.

**Figure 2. Functional validation of macaque CCK promoters in mouse.**

**A** (top) Sequence similarity of upstream regions between mouse and macaque. (bottom) Schematic representation of isolated promoters. **B, D** One week after injection of LVV-CCK-0.5 kb::Venus (B) or LVV-CCK-3.9 kb::Venus (D), transgene expression was analyzed immunohistochemically. Scale bars = 200 μm (low magnification), 20 μm (high magnification). **C, E** Colocalization rate of CCK and Venus after infection with LVV-CCK-0.5 kb::Venus (C) or LVV-CCK-3.9 kb::Venus (E). n = 3 mice.

**Figure 3. Functional validation of macaque PV promoters in mouse.**

**A** (top) Sequence similarity of upstream regions between mouse and macaque. (bottom) Schematic representation of isolated promoters. **B, D** One week after injection of LVV-PV-0.8 kb::Venus (B) or LVV-PV-1.8 kb::Venus (D), transgene expression was analyzed immunohistochemically. Scale bars = 200 μm (low magnification), 20 μm (high magnification). **C, E** Colocalization rate of PV and Venus after infection with LVV-PV-

0.8 kb::Venus (C) or LVV-PV-1.8 kb::Venus (E). n = 3 mice.

**Figure 4. Functional validation of macaque SERT promoters in mouse.**

**A** (top) Sequence similarity of upstream regions between mouse and macaque. (bottom) Schematic representation of isolated promoters. **B, D** One week after injection of LVV-SERT-0.5 kb::Venus (B) or LVV-SERT-1.9 kb::Venus (D), transgene expression was analyzed immunohistochemically. Scale bars = 200 μm (low magnification), 20 μm (high magnification). **C, E** Colocalization rate of TPH2, a marker for serotonin neurons, and Venus after infection with LVV-SERT-0.5 kb::Venus (C) or LVV-SERT-1.9 kb::Venus (E). n = 3 mice.

**Figure 5. Functional validation of macaque vAChT promoters in mouse.**

**A** (top) Sequence similarity of upstream regions between mouse and macaque. (bottom) Schematic representation of isolated promoters. **B, D** One week after injection of LVV-vAChT-1.1 kb::Venus (B) or LVV-vAChT-1.8 kb::Venus (D), transgene expression was analyzed immunohistochemically. Scale bars = 200 μm (low magnification), 20 μm (high magnification). **C, E** Colocalization rate of ChAT, a marker for acetylcholine neurons, and Venus after infection with LVV-vAChT-1.1 kb::Venus (C) or LVV-vAChT-1.8 kb::Venus (E). n = 3 mice.

**Figure 6. Functional validation of macaque SP promoters in mouse.**

**A** (top) Sequence similarity of upstream regions between mouse and macaque. (bottom)

Schematic representation of isolated promoters. **B, D** One week after injection of LVV-SP-0.8 kb::Venus (B) or LVV-SP-1.7 kb::Venus (D), transgene expression was analyzed immunohistochemically. Scale bars = 200 μm (low magnification), 20 μm (high magnification). **C, E** Colocalization rate of SP and Venus after infection with LVV-SP-0.8 kb::Venus (C) or LVV-SP-1.7kb::Venus (E). n = 3 mice.

**Figure 7. Functional validation of macaque PENK promoters in mouse.**

**A** (top) Sequence similarity of upstream regions between mouse and macaque. (bottom) Schematic representation of isolated promoters. **B, D** One week after injection of LVV-PENK-0.9 kb::Venus (B) or LVV-PENK-2.2 kb::Venus (D), transgene expression was analyzed immunohistochemically. Scale bars = 200 μm (low magnification), 20 μm (high magnification). **C, E** Colocalization rate of PENK and Venus after infection with LVV-PENK-0.9 kb::Venus (C) or LVV-PENK-2.2kb::Venus (E). n = 3 mice.