

## Targeted gene expression in dopamine and serotonin neurons of the mouse brain

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### Abstract

We used a knock-in strategy to generate two lines of mice expressing Cre recombinase under the transcriptional control of the dopamine transporter promoter (DAT-cre mice) or the serotonin transporter promoter (SERT-cre mice). In DAT-cre mice, immunocytochemical staining of adult brains for the dopamine-synthetic enzyme tyrosine hydroxylase and for Cre recombinase revealed that virtually all dopaminergic neurons in the ventral midbrain expressed Cre. Crossing DAT-cre mice with ROSA26-stop-lacZ or ROSA26-stop-YFP reporter mice revealed a near perfect correlation between staining for tyrosine hydroxylase and  $\beta$ -galactosidase or YFP. YFP-labeled fluorescent dopaminergic neurons could be readily identified in live slices. Crossing SERT-cre mice with the ROSA26-stop-lacZ or ROSA26-stop-YFP reporter mice similarly revealed a near perfect correlation between staining for serotonin-synthetic enzyme tryptophan hydroxylase and  $\beta$ -galactosidase or YFP. Additional Cre expression in the thalamus and cortex was observed, reflecting the known pattern of transient SERT expression during early postnatal development. These findings suggest a general strategy of using neurotransmitter transporter promoters to drive selective Cre expression and thus control mutations in specific neurotransmitter systems. Crossed with fluorescent-gene reporters, this strategy tags neurons by neurotransmitter status, providing new tools for electrophysiology and imaging.

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### 1. Introduction

Aberrant functioning of the ascending dopaminergic or serotonergic systems is prominent in many neuropsychiatric disorders including schizophrenia, Parkinson's disease, attention deficit hyperactivity disorder, drug addiction, depression, and anxiety. A striking feature of these systems is that they arise from just a few hundred thousand neurons concentrated in brainstem nuclei sending projections to widely distributed target areas (Cooper et al., 1996). We have taken advantage of the dopamine transporter (DAT) and serotonin

transporter (SERT), known specific markers of dopaminergic and serotonergic neurons, respectively (Augood et al., 1993; Hensler et al., 1994), and used their promoters to generate cell-specific Cre recombinase expressing mice.

The Cre-loxP system has become a standard approach for performing region-specific gene inactivation in mice (Kuhn and Torres, 2002). However, this strategy has not previously been used to generate gene knockouts in specific neurotransmitter systems. Traditionally, transgenic lines are generated via pronuclear injection of the transgene that will be integrated into the genome randomly. The level and pattern of transgene expression are determined both by the promoter and by its integration site in the genome. Consequently, such transgenic mice often have transgene expression in regions or cell types not associated with the original specificity of the promoter (Bronson et al., 1996; Wallace et al., 2000). In

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order to direct the expression of the Cre recombinase more precisely to specific monoaminergic cell types, we used a knock-in approach to place the transgene downstream of the endogenous promoters of DAT and SERT.

Mice engineered using such an approach express Cre recombinase in dopaminergic and serotonergic neurons. Crossing these cre mice with reporter lines, demonstrated the high efficiency and specificity of this approach. Moreover, these Cre-expressing lines were used successfully to label dopamine and serotonin neurons in vivo with yellow fluorescent protein (YFP), allowing for visualization of the living neurons in brain slices. Our results represent the first example in which Cre recombinase activity is restricted to a specific monoamine neurotransmitter system and provide a general strategy that can be used to target mutations and fluorescent markers to specific neurotransmitter systems.

## 2. Materials and methods

### 2.1. Generation of transgenic mice

DAT and SERT genomic DNA fragments that contained the 5'-region and the first two exons were excised from phage DNA isolated from a mouse 129 Sv/J genomic library. The gene targeting vectors were constructed by inserting into the 5'-UTR region of the DAT and SERT genes a cassette containing the Cre recombinase coding sequence with a nuclear localization signal and the neomycin-resistance gene, flanked by FRT sites (Fig. 1a and c). W9.5 embryonic stem (ES) cells were electroporated (Bio-Rad Gene Pulse; 800 V and 3  $\mu$ F) with 30  $\mu$ g of linearized targeting construct. G418-resistant clones were screened by Southern blot for homologous recombination with external probes. Positive cells were injected into C57BL/6J blastocysts to generate chimeras. Chimeras were mated with 129 Sv/J females to generate heterozygous mutants on a 129 Sv/J genetic background.

In DAT-cre mice, our initial immunocytochemical study of Cre recombinase expression indicated very low levels of expression in both ventral tegmental area (VTA) and substantia nigra pars compacta (SNc). We reasoned that the PGK-neo-pA selection marker in the targeting cassette might interfere with Cre expression (Fiering et al., 1995; Olson et al., 1996). We, therefore, crossed these mice with the germline deleter ACTB:FLP-L (Dymecki, 1996). Genotyping of tail DNA revealed that all DAT-cre; ACTB:FLP-L double transgenic mice had 20–100% FLP recombinase-mediated deletion of the FRT-flanked PGK-neo-pA cassette. We crossed these double transgenic mice with wild-type mice and found one male out of 26 DAT-cre positive but ACTB:FLP-L negative mice with germline transmission of FLP recombinase-mediated deletion of PGK-neo-pA. We bred this male with wild-type female mice and established a DAT-cre line free of PGK-neo. In SERT-cre mice, high Cre expression was achieved with the presence or absence of PGK-neo-pA cassette which is in an antisense orientation (Fig. 1c).

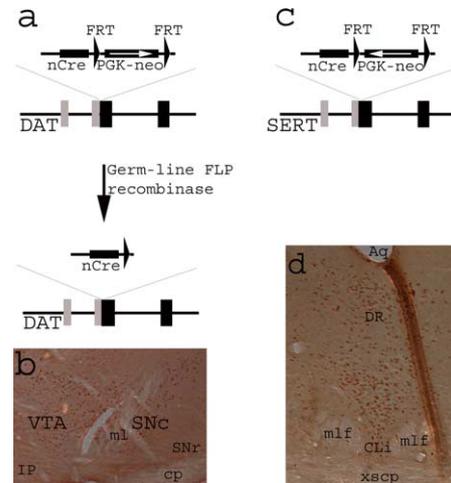


Fig. 1. Dopamine or serotonin neuron specific expression of the Cre recombinase after knocking-in cre to the DAT or SERT locus. (a) DAT-cre targeting construct. A cassette that contains cre, the FRT-flanked neomycin-resistance gene (PGK-neo-pA) was inserted into the 5'-UTR of DAT genomic DNA immediately upstream of the DAT translational start codon. The FRT-flanked PGK-neo-pA was deleted in germline by crosses with the ACTB:FLP-L deleter line. (b) High Cre-recombinase expression in the VTA and SNc in DAT-cre mice. (c) SERT-cre targeting construct. A cassette that contains cre, the FRT-flanked neomycin-resistance gene (PGK-neo-pA) was inserted into the 5'-UTR of SERT genomic DNA immediately upstream of the SERT translational start codon. PGK-neo-pA was inserted in an antisense orientation. (d) High Cre-recombinase expression in the Raphe in SERT-cre mice. Aq, aqueduct; CLi, caudal linear raphe; cp, cerebral peduncle; DR, dorsal raphe; IP, interpeduncular nucleus; ml, medial lemniscus; mlf, medial longitudinal fasciculus; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; VTA, ventral tegmental area; xscp, decussation of superior cerebellar peduncle.

The ACTB:FLP-L germline deleter was kindly provided by Dr. Susan Dymecki, Harvard Medical School. ROSA26-stop-lacZ reporter mice (Soriano, 1999) were purchased from the Jackson Lab. ROSA26-stop-YFP reporter mice (Srinivas

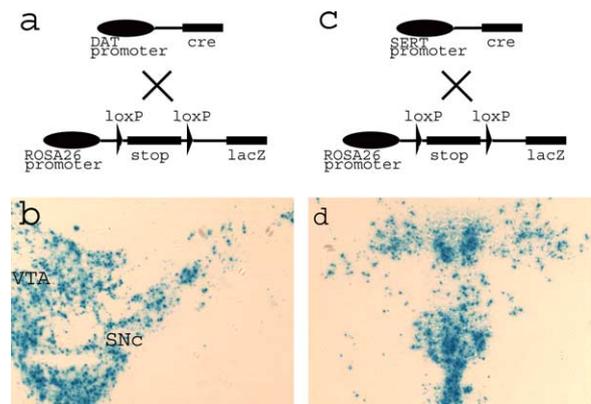


Fig. 2. Cre recombinase activity indicated by the ROSA26-stop-lacZ reporter. (a and b) In DAT-cre, ROSA26-stop-lacZ double transgenic mice, Cre recombinase activity in dopamine neurons deleted the floxed stop sequence, conferring specific  $\beta$ -galactosidase expression in VTA and SNc dopaminergic neurons. (c and d) In SERT-cre, ROSA26-stop-lacZ double transgenic mice, Cre recombinase activity in serotonin neurons conferred  $\beta$ -galactosidase expression in Raphe serotonergic neurons.

et al., 2001) were kindly provided by Dr. Frank Costantini, Columbia University. All animal procedures followed NIH Guidelines and were approved by the IACUC Committees of New York State Psychiatric Institute and Columbia University.

## 2.2. Immunocytochemistry

Adult mice were deeply anesthetized and then perfused with 4 °C saline followed by 4% paraformaldehyde (PFA). Brains were removed and post-fixed overnight in 4% PFA. After washing, brains were cryoprotected in 30% sucrose, and free-floating cryostat sections (40  $\mu$ m) cut. Sections were permeabilized and blocked with 0.1% Triton X-100/10% donkey serum in PBS. Primary antisera were applied overnight at 4 °C with slow agitation. Secondary antibodies were applied at room temperature for 1 h. For single labeling, we used biotinylated secondary antibody at 1:200 (Vector), ABC kit (Vector) and DAB (Sigma). For double labeling, we used fluorescein or rhodamine secondary antisera at 1:200 (Chemicon, Temecula, CA). Dilutions for primary antibodies were: anti-Cre (polyclonal, Novagen) 1:5000; anti-tyrosine hydroxylase (monoclonal, Calbiochem) 1:2000; anti-tryptophan hydroxylase (monoclonal, Chemicon) 1:400. When YFP was used as a second label, YFP fluorescence was visualized directly in fixed sections using a YFP filter set (Omega XF104).

## 2.3. Visualization of $\beta$ -galactosidase activity

Animals were sacrificed by cervical dislocation, brains were quickly removed and frozen on dry ice. Frozen sections (20  $\mu$ m) were cut on a cryostat and fixed in 4% PFA in PBS for 15 min, incubated in X-gal solution at room temperature overnight, and then washed with PBS. X-gal solution had the following composition: 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 2 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 0.037% X-gal.

## 2.4. Imaging

Epifluorescence images were acquired with a chilled CCD digital camera (Photometrics Sensys and Scanalytics IP-Lab software). Color merges were made by placing the individual 8 bit monochrome images in the red or green red–green channels of 24 bit color images. Plates were made using Adobe Photoshop and Macromedia FreeHand.

## 3. Results

### 3.1. Cre recombinase expression in dopaminergic and serotonergic neurons in DAT-cre and SERT-cre mice, respectively

In order to direct the expression of the Cre recombinase precisely to dopaminergic and serotonergic neurons, the cre

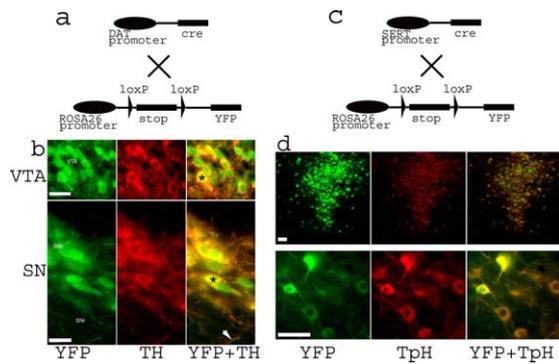
transgene was inserted downstream of the endogenous promoter of DAT (DAT-cre mice) and SERT (SERT-cre mice). Fig. 1a and c illustrate the gene-targeting constructs. Immunohistochemical staining of adult brain sections revealed Cre recombinase expression in the VAT and SNc in DAT-cre mice and in the Raphe nuclei in SERT-cre mice (Fig. 1b and d). In addition to midbrain dopaminergic neurons, weak staining of Cre-positive neurons were also seen in the olfactory bulb and in the hypothalamus (data not shown). These cells correspond to recognized dopaminergic groups (Cooper et al., 1996).

### 3.2. Cre recombinase activity in dopaminergic and serotonergic neurons in DAT-cre and SERT-cre mice, respectively

The above results establish that Cre recombinase expression is indeed restricted to regions of dopaminergic neurons in DAT-cre mice and serotonergic neurons in SERT-cre mice. In order to assess whether Cre recombinase activity was sufficiently robust and restricted to dopaminergic and serotonergic neurons in their respective lines, we crossed both lines with ROSA26-stop-lacZ reporter mice (Fig. 2a and c) (Soriano, 1999). The ROSA26 promoter conveys ubiquitous expression to any downstream gene. The transcription of downstream genes is activated through the action of Cre recombinase to remove a transcriptional “stop” sequence. In DAT-cre, ROSA26-stop-lacZ double transgenic mice, cells in the VTA and SNc stained positively for  $\beta$ -galactosidase (Fig. 2b). In addition, weak lacZ expression was also seen in dopaminergic neurons in the olfactory bulb and in the hypothalamus, corresponding to dopaminergic neurons found in these structures (Cooper et al., 1996) (data not shown). In SERT-cre, ROSA26-stop-lacZ double transgenic mice, lacZ staining was seen in the Raphe nuclei (Fig. 2d), in keeping with the Cre recombinase expression revealed in immunocytochemical studies. However, strong lacZ expression was also seen in the thalamus, cingulate cortex and the CA3 region of the hippocampus (not shown, however, similar results are obtained with the ROSA26-stop-YFP reporter, see Fig. 4). Since these regions were Cre negative and tryptophan hydroxylase (Tph) negative in adult brains, lacZ expression in these regions was likely the result of transient expression of Cre recombinase during development. Indeed, transient expression of SERT in this pattern during early postnatal development has been previously described (Lebrand et al., 1998).

### 3.3. In vivo labeling of dopaminergic and serotonergic neurons by YFP

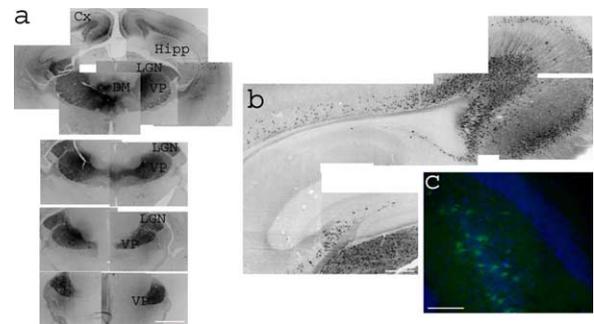
In addition to crossing DAT-cre and SERT-cre mice with ROSA26-stop-lacZ reporter mice, we also crossed DAT-cre and SERT-cre mice with ROSA26-stop-YFP reporter mice (Srinivas et al., 2001), which provided a strategy to fluorescently label dopaminergic and serotonergic neurons in vivo. In DAT-cre, ROSA26-stop-YFP double transgenic



**Fig. 3.** In vivo labeling of dopaminergic and serotonergic neurons by YFP. (a and b) In DAT-cre, ROSA26-stop-YFP double transgenic mice, Cre recombinase activity in dopamine neurons deleted the floxed stop sequence, allowing expression of YFP in VTA and SNc dopamine neurons. YFP fluorescence is shown in green (left panels), TH immunolabeling is in red (center panels) and superimposition of the two is shown in yellow (right panels). The stars show examples of double-labeled cells. The arrow shows an example of a double-labeled process. (c and d) In SERT-cre, ROSA26-stop-YFP double transgenic mice, YFP fluorescence is shown in green (left panels), TpH immunolabeling is in red (center panels) and superimposition of the two is shown in yellow (right panels). Calibrations: (b) 20  $\mu\text{m}$ ; (d) 40  $\mu\text{m}$ .

mice, YFP fluorescence demonstrated a high degree of efficiency and specificity in its overlap with subsequent tyrosine hydroxylase (TH) staining (Fig. 3b). Virtually all TH-positive cells (i.e. dopaminergic neurons) were YFP-positive. Very occasional YFP-positive but TH-negative cells were seen in the cortex, septum and hippocampus (not shown). In double-immunostained sections, 92% of YFP-labeled neurons ( $n = 103$ ) stained for TH in the VTA, and 97% ( $n = 175$ ) in the SN. Overall, 95% of YFP-labeled neurons ( $n = 274$ ) stained for TH and were, therefore, identified as dopaminergic neurons.

In SERT-cre, ROSA26-stop-YFP double transgenic mice, YFP fluorescence was seen in the Raphe (Fig. 3d), thalamus (especially in the ventral posterior thalamus, dorsomedial thalamus, and lateral geniculate nucleus) cingulate cortex and hippocampal pyramidal cells (Fig. 4). In the Raphe, YFP fluorescence overlapped completely with TpH staining (Fig. 3d). Virtually all TpH-positive cells (i.e. serotonergic neurons) were YFP-positive. In double-immunostained sections through the Raphe, 99% of YFP-labeled neurons ( $n = 410$ ) stained for TpH, and were, therefore, identified as serotonergic neurons. Overall, SERT-cre mice seem to label serotonin neurons better than DAT-cre mice label dopamine neurons based on the overlap between YFP and respective serotonin or dopamine neuron markers. However, a more extensive study is needed to reach a definitive conclusion. Thalamus, cingulate cortex and hippocampus are regions devoid of SERT expression in adulthood that have YFP expression in adult SERT-cre/ROSA26-YFP mice due to transient SERT expression during late embryonic and early postnatal development. These same expression patterns have been reported (Lebrand et al., 1998).



**Fig. 4.** Broad distribution of YFP+ cells in SERT-cre/ROSA26-YFP mouse in thalamus, cortex and hippocampus. (a) Coronal sections (rostral-most section on top), showed YFP labeling in the deep and retrosplenial granular layer in cortex (Cx), hippocampus (Hipp), ventral posterior thalamus (VP), dorsomedial thalamus (DM), and the lateral geniculate nucleus (LGN). (b) At higher magnification, dense YFP expression is evident in retrosplenial cortex and in the pyramidal cell layer. (c) At still higher magnification, with DAPI (blue) as a counterstain to reveal all nuclei, selective staining of a subset of CA4 pyramidal neurons (green) is evident. In contrast, no YFP expression is seen in the dentate granule cells. Calibrations: (a) 250  $\mu\text{m}$ ; (b) 100  $\mu\text{m}$ ; (c) 50  $\mu\text{m}$ .

#### 4. Discussion

The promoters of the serotonin and dopamine transporters were used to drive Cre recombinase expression in order to achieve precise neuron-specific Cre recombinase activity. The most important application of these DAT-cre and SERT-cre mice will be to target mutations specifically to dopaminergic and serotonergic neurons, respectively. For example, DAT-cre mice will allow us to perform knockout of dopamine D2 receptors specifically in dopamine neurons (i.e. D2 autoreceptor specific knockout). In addition, by crossing dopaminergic neuron Cre mice or serotonergic neuron Cre mice with reporter mice, our results indicate that a neurotransmitter-specific Cre line can also be used to label neurons of a specific neurotransmitter system with  $\beta$ -galactosidase or a fluorescent marker such as YFP. Mice with dopaminergic or serotonergic neuron specific fluorescent markers are becoming important tools for electrophysiological recordings from identified neurons in the brain slice (Chuhma et al., 2004). They may also serve for the isolation of dopaminergic or serotonergic neurons by fluorescence-activated cell sorting. Recently, a rat tyrosine hydroxylase promoter driven Cre line was generated which has Cre expression in catecholaminergic neurons (Gelman et al., 2003). However, our results represent the first example in which Cre recombinase activity is restricted to a specific monoamine neurotransmitter system.

Considered broadly, this genetic approach provides a general strategy for neurotransmitter-specific gene targeting by using the endogenous neurotransmitter transporter promoter to direct Cre recombinase expression. Compared to traditional transgenic approach with random integration of transgenes, knock-in approaches have a number of advantages. First, the expression pattern is much more accurate.

Second, it circumvents the necessity of isolating and characterizing the promoter, which is usually a lengthy process. Third, the inserted transgene will not create an additional insertional mutation at an unknown locus. One main concern of the 5'-UTR knock-in approach is that it will disrupt one copy of the gene. Both SERT heterozygous knockout mice (Fabre et al., 2000; Gobbi et al., 2001; Holmes et al., 2002) and DAT heterozygous knockout mice (Giros et al., 1996; Spielewoy et al., 2000; Savelieva et al., 2002; Hall et al., 2003) show subtle behavioral and biochemical changes compared to wild-type mice. However, under certain behavioral or pharmacological challenges, the difference between wild-type animals and heterozygous mutants could be important. For example, heterozygous DAT knockout mice develop pronounced locomotor sensitization by amphetamine (Spielewoy et al., 2001). Even though this potential problem may be minimized by using appropriate control mice (so that all experiments will be performed on mice with one copy of DAT or SERT), experiments should be interpreted carefully.

Viral vector mediated Cre recombinase expression in specific brain regions have also been used extensively in recent years (e.g. Scammell et al., 2003; South et al., 2003). An important advantage of viral vectors is that they do not require a region-specific promoter. However, in many cases, cell specificity is very important (for example, viral vector injection into the midbrain will not ensure dopamine neuron specific expression). In addition, viral vector injections have to be made to each animal, which is not the case for transgenic lines.

Interestingly, the selection marker PGK-neo significantly inhibited Cre expression in the DAT-cre construct (good expression was achieved only after the removal of PGK-neo) but not in the SERT-cre construct. The only difference between the two constructs besides their different loci was that PGK-neo was inserted in a sense orientation in the DAT-cre construct and in an antisense orientation in the SERT-cre construct. Even though genetic studies have documented that PGK-neo can inhibit gene expression when it is placed in the promoter region or in the intron (Ley et al., 1998; Meyers et al., 1998), there has been no systematic study on the significance of the location and/or orientation of PGK-neo on the expression level and/or pattern of a neighboring gene. This is to our knowledge the first example in which PGK-neo placed downstream of a cDNA and its polyA sequence in a sense orientation (but not in an antisense orientation) reduce the cDNA expression level without altering the expression pattern. Whether this is a universal phenomena remains to be investigated.

Importantly,  $\beta$ -galactosidase expression in the SERT-cre; ROSA26-stop-lacZ double transgenic adult mice or YFP expression in the SERT-cre; ROSA26-stop-YFP double transgenic adult mice is much more wide spread than Cre recombinase expression pattern found in the adult. This is due to transient expression of SERT during early postnatal development (Lebrand et al., 1998). Since Cre recombinase-mediated DNA excision is irreversible, Cre activity at anytime during

development will recombine the reporter allele and be reflected in  $\beta$ -galactosidase or YFP expression in adulthood. These double transgenic mice give us access to the fate of the transient SERT expressing population. In addition, these results indicate that Cre expression in the thalamus could be useful to target the thalamocortical neurons that transiently express SERT in early postnatal development. However, this line will not be suitable for tissue-specific knockout restricted to Raphe serotonin neurons. In addition, it is very well documented that SERT is expressed in the gut and in chromaffin cells (Chen et al., 2001; Schroeter et al., 1997). Whether SERT-cre mice can delete loxP flanked DNA sequences in these tissues as well as other peripheral tissues will need to be addressed in future studies.

Taken together, these results demonstrate the utility and importance of new tools to control gene expression in two of the primary monoaminergic neuronal subtypes in mice. Moreover, this approach provides a strategy for targeting genetic mutations and fluorescent markers to neurotransmitter systems more generally.

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