Targeting Cre Recombinase to Specific Neuron Populations With Bacterial Artificial Chromosome Constructs

Supplementary Material

Supplemental METHODS

BAC transgene construction

The BAC transgenic vector was prepared as previously report (Gong et al., 2003). The "A box" was amplified from the sequence right before the Start codon. After the homologous recombination through A homology arms, the endogenous messenger RNA and protein coding sequences have been replaced by the sequences encoding the Cre recombinase. The expression pattern of Cre recombinase will mirror that of the starting gene when reintroduced into transgenic animals.

SUPPLEMENTAL FIGURE 1

Supplemental Figure 1. Diagram of the generation of the BAC-Cre construct using a shuttle vector system to insert Cre into the BAC construct. In Step 1, a 300-400 base pair sequence of the region immediately preceding the ATG start codon sequence of gene X (designated the A Box), is cloned from a BAC containing the gene, and is inserted into the shuttle vector adjacent to the coding sequence for Cre recombinase (Cre-I-Cre polyA). In STEP 2, the shuttle vector is used to insert the Cre sequence into the BAC containing gene X.

Tamoxifen inducible Cre expression

In order to induce ligand-dependent site-specific recombination in mice, we cloned the Cre-ERT2 into our shuttle vector, which is insensitive to the natural ligand 17 B-oestradiol (E2), and is highly sensivite to the synthetic oestrogen antagonists tamoxifen, 4-hydroxytamoxifen (OHT) and ICI 182,780 (ICI). The plasmid pLD53SC-Cre-ERT2 is also derived from the pLD53.SC. A 2 Kb fragment containing Cre-ERT2 and a 174 bp fragment containing SV40 polyA were cloned into its SmaI/SalI sites. This shuttle vector (pLD53.SC-CreERT2) was digested with AscI/SmaI and purified by running on a 1% low-melting agarose gel. A 300-500 bp "A box" fragment was amplified from BAC clone by polymerase chain reaction was cloned into AscI/SmaI sites.

Mice generated with tamoxifen inducible BAC-Cre (BAC-TI-Cre) constructs were crossed with ROSA26-EGFP transgenic mice. Offspring that contained both the ROSA and BAC-TI-Cre constructs were housed under normal conditions for 2 months, and then treated with tamoxifen. Tamoxifen was prepared by first dissolving in ethanol (60 mg/100 µl) and mixing this solution with 900 µl corn oil for a final concentration of 60 mg/ml. Mice (weighing approximately 30 g) were injected with 100 µl tamoxifen solution either once on one day or 4 times over 5 days (2 days of injection, one day off, and 2 days of injection). Animals were monitored for adverse effects, and if these became apparent, treatment was stopped. One week after treatment was ended, animals were processed as described below for localization of EGFP.

Characterization of Cre expression

Transgenic animals containing BAC-Cre constructs were mated to ROSA26-EGFP transgenic mice (Soriano, 1999). These mice contain a transgene containing loxP sites flanking a stop codon inserted between the ROSA26 promoter and the coding sequence for enhanced green fluorescent protein (EGFP). Offspring were genotyped between 21 and 28 days and those containing both the Cre and ROSA-EGFP constructs were deeply anesthetized and perfused with normal saline followed by phosphate buffered formaldehyde (4%). Following overnight fixation, brains were immersed in 20% sucrose, and frozen sectioned at 40µm. Sections were processed for immunohistochemical localization of EFGP using standard techniques. Antibodies to EGFP (rabbit anti EGFP) were used at a dilution of 1:1000 and visualized using either avidin-biotin peroxidase complex (ABC, Vector Chemical) to produce a diaminobenzidine (DAB) histochemical reaction. In some cases EGFP was co-localized with a second antigen, including choline acetyltransferase (mouse anti-ChAT), tyrosine hydroxylase (mouse anti-TH), tryptophan hydroxylase (mouse anti-TPH) or 2'3'-cyclic nucleotide 3'-phosphodiesterase (mouse anti-CNP, Chemicon

#MAB326R), with both antigens being visualized with fluorescent secondary antibodies to determine co-expression.

GENE **BAC-Cre transgenic line(s)**	**BAC clone**	**Cre expression pattern** **Adult / developmental / partial**
Choline Aceytl Transferase (ChAT)	RP23-246B12	
GM 24 / GM 60		Adult
GM 53		partial : motor nuclei only
Tyrosine Hydroxylase (TH)	RP23-350E13	
FI 12 / FI 172		Adult plus developmental pattern
Serotonin Transporter (slc6a4)	RP24-335M24	
ET 33 / ET 35		Adult
ET 124		Adult plus developmental pattern
Noradrenalin Transporter (slc6a2)	RP23-109O23	
FV 317 / FV 319		Adult
ETS domain transcription factor (etv1)	RP23-250K4	
GM 225		Neocortex layer 5
Neurotensin Receptor 1 (NTSR1)	RP23-314D14	
GN 220		Neocortex layer 6
D2 dopamine receptor (drd2)	RP23-161H15	
ER44		Adult
ER43		Partial: striatopallidal neurons
D1 dopamine receptor (drd1a)	RP23-47M2	
EY262/FK161/FK150		Adult
Purkinje cell protein (pcp2)	RP24-186D18	
GN135		Adult
oligodendrocyte protein (cmtm5, cklfs5)	RP24-317F19	
GM160		Adult

Table 1: List of BAC-Cre lines described in this study

Supplemental RESULTS

Neurotransmitter specific genes.

Tyrosine hydroxylase is a key enzyme in the catecholamine synthetic pathway and is expressed in neurons that utilize both dopamine and noradrenalin as neurotransmitters. These neurons are distributed in disCrete nuclei including specific nuclei in the hypothalamus, midbrain, hindbrain and brain stem nuclei (Lindvall and Bjorklund, 1983). Two TH BAC-Cre lines were generated in which Cre expression was localized to most of the TH containing neurons in the brain, as demonstrated with co-expression of EGFP and TH-immunoreactivity. Notably, over 90% of the TH neurons of the midbrain dopamine neurons, which provide projections to the forebrain, including the striatum and cortex (Figure 1A). Also well labeled were neurons in the locus coeruleus, in which are located neurons utilizing noradrenalin as a neurotransmitter, and which project broadly to the forebrain. In addition, in the forebrain, there were a number of neurons in which Cre was expressed that did not express TH (supplemental figure 2). These

neurons are most likely those which have been shown to express TH transiently during development, but do not express TH in the adult (Saino-Saito et al, 2003; Marin et al. 2005).

SUPPLEMENTAL FIGURE 2

Supplemental Figure 2. An example of ectopic Cre expression resulting from transient expression during development is produced in tyrosine hydroxylase (TH) BAC-Cre transgenic lines (F12). This TH BAC-Cre line produces Cre expression in all dopaminergic and noradrenergic neurons in the adult (see figure 3). In addition, this line also shows expression in neurons in various brain areas, which do not express TH in the adult. Shown is EGFP labeling generated by Cre expression in the hypothalamic area of the zona incerta, in which there are neurons that co-express EGFP and TH immunoreactivity (orange arrows), as well as neurons that are EGFP immunoreactive but TH immunoreactive negative (blue arrows). These latter neurons are likely those which express TH transiently during development but not in the adult.

The serotonin transporter (solute carrier family 6, member 4, slc6a4) is expressed specifically in adult neurons that utilize serotonin as a neurotransmitter (Hansson et al., 1998; Lebrand et al., 1998). Serotonin neurons are located in disCrete nuclei in the hindbrain and brainstem (Steinbusch and Mulder, 1984). Two slc6a4 BAC-Cre lines (ET33, ET35) displayed expression localized to neurons of the serotonin neuron cell groups, including the dorsal and median raphe (Figure 3C). Double labeling shows that nearly all EGFP labeled neurons in the raphe co-express tryptophan hydoxylase, which is a specific marker for serotonin neurons. Labeling in other parts of the brain is minimal in these lines, with only a few scattered neurons in layer 6 of the cingulated cortex and in the ventrolateral thalamus.

Whereas in the adult the serotonin transporter is specifically expressed in serotonin neurons, during development there is transient expression in forebrain neurons, which are not serotonergic (Hansson et al., 1998, Lebrand et al., 1998). Another slc6a4 BAC-Cre line (ET124) displayed expression likely due to such transient developmental expression. In this line labeling included, layer 6 neurons in the cerebral cortex as well as thalamocortical neurons. Cortical areas in which neurons are labeled are extensive, including most of the rostral areas, sensory, motor as well as cingulate and subicular cortices. Labeled thalamic neurons were restricted to sensory relay nuclei, including the ventral posterior nuclei as well as the dorsal and lateral geniculate nuclei. In addition, some lines also show expression in the hypothalamus. None of the EGFP labeled neurons in these slc6a4 BAC-Cre transgenic mice that are not in nuclei containing serotonin neurons in the hindbrain and brainstem, expressed tryptophan hydroxylase and are thus presumed to be non-serotoninergic.

SUPPLEMENTAL FIGURE 3

Supplemental Figure 3. Example of ectopic Cre expression resulting from transient developmental expression is demonstrated in a comparison of two serotonin transporter (slc6a4) BAC-Cre lines (ET33 and ET124). The ET33 slc6a4 BAC-Cre transgenic line produces Cre expression that is restricted to serotonin neurons in the dorsal and median raphe nuclei, which is the pattern of expression of slc6a4 in the adult animal. On the other hand the ET124 slc6a4 BAC-Cre transgenic line produces expression in layer 6 corticothalamic neurons, in thalamocortical neurons, as well as in the serotonin neurons in the dorsal and median raphe nuclei. Expression in corticothalamic and thalamocortical is likely due to transient expression of slc6a4 during development, which produces Cre expression that persists in adult animals.

Neurons utilizing acetyl choline as a neurotransmitter express ChAT and are distributed in distinct neuron populations throughout the brain (Armstrong et al, 1983; Lauterborn et al, 1993), which are labeled in a BAC-EGFP transgenic line (Gong et al., 2003) Transgenic BAC-Cre transgenic lines (GM24 and GM60) generated with the same BAC clone express Cre, which matches eutopic ChAT expression as demonstrated with the Rosa26 EGFP reporter (Figure 2). In the forebrain EGFP expression is present in neurons in the cerebral cortex, striatum and basal forebrain. In the cerebral cortex, labeled neurons, with a bipolar dendritic morphology, are located in layers 2-3 in neocortical regions including somatosensory and motor areas. In the striatum, labeled neurons are scattered, with a large cell body and smooth dendrites corresponding to the large aspiny cholinergic neuron of the striatum. Also labeled in the forebrain are neurons comprising the basal forebrain cholinergic neuron population, which provides projections to the cerebral cortex, including neurons located in the medial septal nucleus, substantia innominata, and ventral globus pallidus. In the midbrain, neurons in the pedunculopontine nucleus were labeled, as were motor

neurons in cranial nerve nuclei, including the trochlear and abducens nuclei. In the brainstem and spinal cord, motor neurons were also labeled. All neurons labeled in these animals were shown to co-express ChAT immunoreactivity. A third ChAT BAC-Cre transgenic line (GM53), displayed EGFP labeling restricted only to motor neurons, both in the cranial nuclei and the spinal cord.

SUPPLEMENTAL FIGURE 4

Supplemental Figure 4. Recombinase (Cre) expression in a transgenic mouse line (GM24) generated with a choline acetyl transferase (ChAT) BAC-Cre construct, visualized by enhanced green fluorescent protein (EGFP) immunohistochemistry in the brains of animals crossed with a Rosa26-EGFP reporter mouse line. Labeling is present in all known cholinergic neurons, throughout the brain and spinal cord. In a coronal section through the forebrain (A), major cholinergic neuron types are labeled, including biopolar neurons in the cerebral cortex (Ai), large aspiny neurons in the striatum (Aii) and cortical projecting neurons in the basal forebrain (B). Labeled basal forebrain neurons are shown to be cholinergic by the co-labeling with EGFP (B', green) and ChAT (B'', red) immuno-histochemistry. All EGFP and ChAT-IR neurons co-express both markers (insets). Cholinergic motor neurons are labeled in the midbrain (C) trochlear motor nucleus (C') and in dorsal horn motor neurons in the spinal cord (D). In the caudal midbrain (E), expression is present in the pedunculopontine nucleus (PPN in Ei) and in motor neurons of the trigeminal nucleus and their axons. In another ChAT BAC-Cre line (GM53) expression is restricted to motor neurons as seen by the absence of labeling in the PPN, while the motor neurons of the trigeminal nucleus show robust expression (F).

The noradrenaline transporter (solute carrier family 6, member 2, slc6a2) in the adult is specifically expressed in noradrenergic neurons and thus provides a marker to distinguish the subset of TH- positive neurons that utilize noradrenalin. BAC-Cre transgenic lines utilizing the slc6a2 gene were generated, which display expression of Cre selectively in the locus coeruleus and lateral dorsal tegmental nucleus. Approximately two thirds of the TH-immunoreactive neurons in these nuclei displayed Cre expression.

SUPPLEMENTAL FIGURE 5

Supplemental Figure 5. Cre expression directed to noradrenergic (C) neurons in BAC-Cre transgenic mouse line. Noradrenergic neurons are labeled in a norardrenaline transporter (Slc6a2) BAC-Cre transgenic line in neurons in the locus coeruleus, which are co-labeled with tyrosine hyroxylase (TH)-IR. Most neurons in the locus coeruleus co-express Slc6a2 Cre-driven EGFP and TH-IR.

Genes expressed in specific forebrain circuits

One drd2 BAC-Cre transgenic line displayed expression that matches that of the drd2 BAC-EGFP line (ER44). The most prominent neuron population labeled is the striatal spiny projection neurons, which make up the "indirect" striatal pathway of the basal ganglia. These neurons are distributed throughout all regions of the striatum including the caudate-putamen and nucleus accumbens and constitute approximately half of the neurons in these regions. Also well labeled are the axonal projections of these neurons, which project to the globus pallidus and ventral pallidal areas. Also very prominently labeled are neurons in the olfactory tubercle, which is the ventral extension of the striatum. In addition to labeling of striatal neurons, other areas in the forebrain in which labeled neurons were present, include the cerebral cortex, septal area, basal forebrain, amygdala, hypothalamus, and thalamus. In the midbrain, labeled neurons include the dopamine neurons in the substantia nigra pars compacta, pretectal nuclei, superior and inferior colliculus as well as neurons in the parabrachial nucleus and other brainstem nuclei. The very specific distribution of labeled neurons in each of these areas matches the expression of the drd2 gene in the brain (Weiner et al., 1991) and that seen in the drd2 BAC-EGFP line.

In a second drd2 BAC-Cre line (ER43), expression is more restricted relative to the first line and the drd2 BAC-EGFP line. As in these lines, there is very prominent labeling of the striatal neuron population, which constitutes the "indirect" striatal pathway, including the labeling of the axonal projections of these neurons to the globus pallidus. In contrast to the first line, there is very limited labeling of neurons outside the striatal indirect pathway system. Within the forebrain, there is essentially no labeling of neurons in the cerebral cortex, hippocampus, septal area, or thalamus, while only a few

scattered neurons being labeled in the basal forebrain. In the midbrain, labeled neurons include the dopamine neurons, but only a small percentage of neurons in other midbrain areas.

SUPLLEMENTAL FIGURE 6

Supplemental Figure 6. Restricted Cre expression produced in a drd2 BAC-Cre transgenic line (ER43) compared with the normal expression of drd2 seen in the drd2 BAC-EGFP transgenic line and in another drd2 BAC-Cre transgenic line (ER44). The normal expression of drd2a is demonstrated in a drd2 BAC-EGFP transgenic line in which there is expression in striatopallidal neurons(2), and additionally in the lateral septal nucleus (1) and in the cortex (3). This pattern of expression is produced in one drd2 BAC-Cre transgenic line (ER44). In another drd2 BAC-Cre transgenic line (ER43), Cre expression is restricted only to striatal neurons providing axonal projections to the globlus pallidus, but not in the lateral septal nucleus or in the cortex. The normal expression of drd2 in dopamine neurons in the substantia nigra pars compacta (SNc) is seen in the drd BAC-EGFP line and in ER44, but not in ER43.

Purkinje cell protein (pcp2) and oligodendrocyte protein (cmtm5) BAC-Cre transgenic lines

CKLF-like MARVEL transmembrane dopamine containing 5 (cmtm5) is a protein expressed in oligodendrocytes. A cmtm5 BAC-Cre line crossed with Rosa26-EGFP reporter produced labeling of cells throughout the brain, which had the morphologic appearance of oligodendrocytes (Figure 8 B). Labeled cells co-express 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP, Figure 8C), which is a specific marker for glial oligodendrocytes (Sprinkle, 1999).

SUPPLEMENTAL FIGURE 7

Supplementary Figure 7. A CKLF-like MARVEL transmembrane containing 5 (cmtm5) BAC-Cre transgenic line produces Cre expression in oligodendrocyte cells throughout the brain. Cells in the white matter of the corpus callosum (cc) in cmtm5 BAC-Cre lines, which are labeled with EGFP-IR are shown to also express cyclic nucleotice phospho-diesterase (CN) immunoreacitivty, which is a specific protein marker for oligodendrocyte glia.

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