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TECHNOLOGY REPORT

Inducible and Neuron-Specific Gene Expression in the Adult Mouse Brain With the rtTA2S-M2 System

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Summary: To achieve inducible and reversible gene expression in the adult mouse brain, we exploited an improved version of the tetracycline-controlled transactivator-based system (rtTA2^S-M2, rtTA2 hereafter) and combined it with the forebrain-specific CaMKIIα promoter. Several independent lines of transgenic mice carrying the CaMKIIα promoter-rtTA2 gene were generated and examined for anatomical profile, doxycycline (dox)-dependence, time course, and reversibility of gene expression using several lacZ reporter lines. In two independent rtTA2-expressing lines, dox-treatment in the diet induced lacZ reporter expression in neurons of several forebrain structures including cortex, striatum, hippocampus, amygdala, and olfactory bulb. Gene expression was dose-dependent and was fully reversible. Further, a similar pattern of expression was obtained in three independent reporter lines, indicating the consistency of gene expression. Transgene expression could also be activated in the developing brain (P0) by dox-treatment of gestating females. These new rtTA2-expressing mice allowing inducible and reversible gene expression in the adult or developing forebrain represent useful models for future genetic studies of brain functions. genesis 43:205–212, 2005. **© 2005 Wiley-Liss, Inc.**

Key words: inducible and reversible gene expression; rtTA2; brain; neurons; doxycycline

Cellular functions recruit multiple specific proteins whose functions often depend on tissue, cell type, or developmental stage. To study these functions in vivo, genetic technologies such as transgenesis and gene knockout were developed in mice for overexpressing, or partially or completely eliminating, selected proteins. To be specific, these manipulations ought to be spatially and temporally controlled for the activity of the protein of interest to be modulated only in selected cells and over a restricted time window. Spatial control is generally achieved by using tissue- or cell-specific promoters and temporal control with ligand-dependent expression systems. The tetracycline-controlled transactivator (tTA)-dependent systems (Gossen and Bujard, 1992) have been widely used for inducible gene expression both in vitro and in vivo. tTA (also called Tet-Off) is a fusion protein between the tetracycline repressor from the Tn10 tetracycline resistance operon of *E. coli* and the C-terminal domain of the transcription factor from *Herpes Simplex Virus* (HSV) VP16. The resulting hybrid transcriptional activator can trigger expression from a cognate promoter made of minimal promoter sequences placed downstream from seven repeats of the tetracycline operator (tetO). It is constitutively active but its activity can be blocked by tetracycline or analogs such as doxycycline (dox) or minocycline. The reverse tTA (rtTA or Tet-On), obtained by random mutagenesis of tTA, has opposite features. It is constitutively silent but needs dox to bind tetO sequences and induce gene transcription (Gossen *et al.,* 1995; Orth *et al.,* 2000).

Both tTA and rtTA factors have been extensively used in transgenic mouse models to achieve dox-controlled gene expression (Morozov *et al.*, 2003; Yamamoto *et al.*, 2001). Although very useful, these systems have several limitations that make their use sometimes difficult in vivo. When coupled to promoters active during early development, the tTA system may need to be turned off by dox treatment to avoid the potential detrimental effects of early transgene expression on general developmental processes. After prolonged dox treatment, transgene induction has a slow kinetic due to poor dox clearance from the body and may not be fully reactivated (Bejar *et al.,* 2002; and own observations). The rtTA system is better in this respect because its kinetic of induction can be faster and transgene expression can be detected in the brain within several days after oral dox treatment (Mansuy *et al.,* 1998) or several hours when

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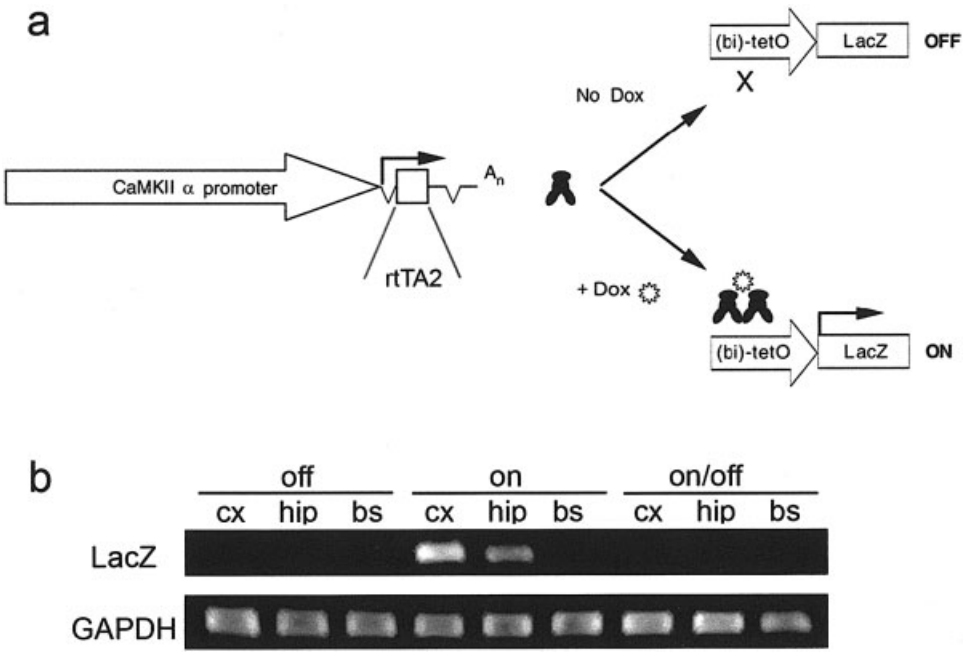


FIG. 1. **a:** Principle of dox-controlled rtTA2 expression system. A first transgene carries the CaMKIIα promoter driving the expression of rtTA2^S-M2 (rtTA2) in forebrain neurons. A second independent transgene carries tet operator sequences (tetO or bitetO) and a lacZ reporter gene encoding β-galactosidase. The bitetO promoter is a bidirectional promoter allowing simultaneous expression of two genes. When bound to dox, rtTA2 switches to an active conformation, which favors binding to tetO sequences (Orth *et al.*, 2000) and induction of gene expression (ON state). In the absence of dox, rtTA2 does not bind to tetO and the transgene is not expressed (OFF state). **b:** Inducibility and reversibility of dox-dependent gene expression (line 898 used as representative). Double transgenic mice (rtTA2 line 898/tetO-lacZ) were fed 6 mg/g dox in food for 8 days (on). For some animals, dox was withdrawn and regular food was provided for 3 weeks (on/off). Double mutant mice not treated with dox (off) were negative controls. LacZ expression was examined by RT-PCR in cortex (cx), hippocampus (hip), and brainstem (bs) with glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) as positive control.

dox is injected stereotactically (our observations). However, rtTA is often less efficient than tTA and leads to lower levels of transgene expression. It also requires higher doses of dox for induction and was sometimes reported to show dox-independent expression in vitro (Giménez *et al.*, 2004; Urlinger *et al.*, 2000).

To improve the use of dox-dependent systems for in vitro and in vivo applications, Urlinger *et al.* (2000) recently developed new versions of the rtTA factor with increased mRNA stability, optimized codon-use for mammals, lower background activity, and higher affinity for dox. We took advantage of one of these factors, rtTA2^S-M2 (rtTA2 hereafter) and adapted it to the adult mouse brain by combination with the forebrain-specific CaMKIIα promoter (Mayford *et al.*, 1996). Several independent lines of CaMKIIα promoter-rtTA2 were generated and crossed with mice carrying a tetO- or bitetO-lacZ reporter gene to evaluate the dox-dependent inducibility and reversibility of gene expression (Fig. 1a).

In two lines of rtTA2-expressing animals (894 and 898), forebrain-specific expression of the lacZ reporter gene was induced by dox treatment (6 mg/g dox for a minimum of 6 days) but none in the absence of dox (off), indicating full dox-dependence of transgene expression (Fig. 1b, not shown for 894). LacZ gene expression could be suppressed in both cortex and hippocampus after dox removal (on/off), showing full reversibility of transgene expression. When induced in the adult brain, β-galactosidase activity was detected in various cortical regions, including hippocampus, striatum, septum, olfactory bulb, and amygdala but not in thalamus, brainstem, or cerebellum, consistent with the endogenous pattern of CaMKIIα expression (Burgin *et al.*, 1990; Herms *et al.*, 1993; Sola *et al.*, 1999; Zou *et al.*, 2002) (Fig. 2). The expression pattern was reproducible across animals and was similar in 894 and 898 lines (not shown for 894). Forebrain-specificity was also consistently observed in two other reporter lines carrying a bidirectional tetO promoter (bitetO) fused to lacZ gene (Baron *et al.*, 1995). When crossed with rtTA2-expressing mice (line 898), these animals showed β-galactosidase expression in most forebrain structures (Figs. 3, 4). The signal, however, was stronger and denser than in the tetO reporter line. For instance, the vast majority of pyramidal cells in hippocampus areas CA1, CA2, CA3, and dentate gyrus expressed lacZ in both bitetO reporter lines, contrasting with the mosaic expression observed in the tetO-lacZ line. Similar heterogeneity in lacZ expression pattern and intensity was observed in cortex, striatum, and amygdala depending on the reporter lines.

A great advantage of the CaMKIIα promoter is its neuronal specificity (Mayford *et al.*, 1996). To evaluate whether this feature was maintained in our rtTA2-

expressing mice (line 898), we performed double immunostaining using antibodies against β-galactosidase and the neuron-specific marker NeuN. Co-staining revealed that all cells expressing lacZ in cortex, hippocampus, amygdala, and other brain structures are positive for NeuN, confirming the neuronal specificity of gene expression (Fig. 5). We also examined the dose-dependence and time course of gene expression. Administration of increasing doses of dox showed that 6 mg/g dox (in wet food, corresponding to 3.47 ± 1.17 mg dox/g body weight/day) induced the strongest lacZ expression, while 3 or 1 mg/g (corresponding to 1.89 ± 0.4 or 0.53 ± 0.07 mg dox/g body weight/day, respectively) induced a more restricted expression, essentially excluding cortical structures (Fig. 6a). Likewise, dox administered in drinking water at 3.5 or 2 mg/ml induced only partial lacZ gene expression but no expression at a lower concentration (0.05 mg/ml) (Fig. 6b). These results indicate that gene expression depends on the dose of dox and that full expression requires 6 mg/g dox in food. The restricted expression obtained with lower doses is notable and may be useful to spatially limit the effect of a transgene. In terms of time course, a gradual increase in lacZ expression was observed after 4, 6, or 8 days of dox treatment (6 mg/g wet food). β-Galactosidase activity was first detected after 4 days (no signal after 2 days), but was less dense and intense than after 6 or 8 days (Fig. 6c). Thus, overall the level of lacZ expression correlated with the amount and duration of dox treatment without any remarkable change in expression pattern.

Because inducible expression systems can be useful to developmental studies, we examined whether gene expression could be induced during embryogenesis in our rtTA2-expressing animals (line 898). We tested an early time point of induction by administering dox to dams through gestation and examined β-galactosidase expression in the brain of newborn pups. Expression was detected in the olfactory bulb, cortex, striatum, and hippocampus and in small areas of midbrain and hindbrain (Fig. 7). Such early expression is consistent with previous data showing prenatal transgene expression using the same CaMKIIα promoter (Krestel *et al.*, 2001; Yamamoto *et al.*, 2000). Prenatal activation is specific to this promoter fragment but does not reflect endogenous CaMKIIα expression, which is essentially postnatal in the mouse and rat brain (Burgin *et al.*, 1990; Herms *et al.*, 1993; Sola *et al.*, 1999; Zou *et al.*, 2002).

In summary, we generated two new lines of transgenic mice expressing rtTA2 under the control of the CaMKIIα promoter that allow inducible and reversible dox-

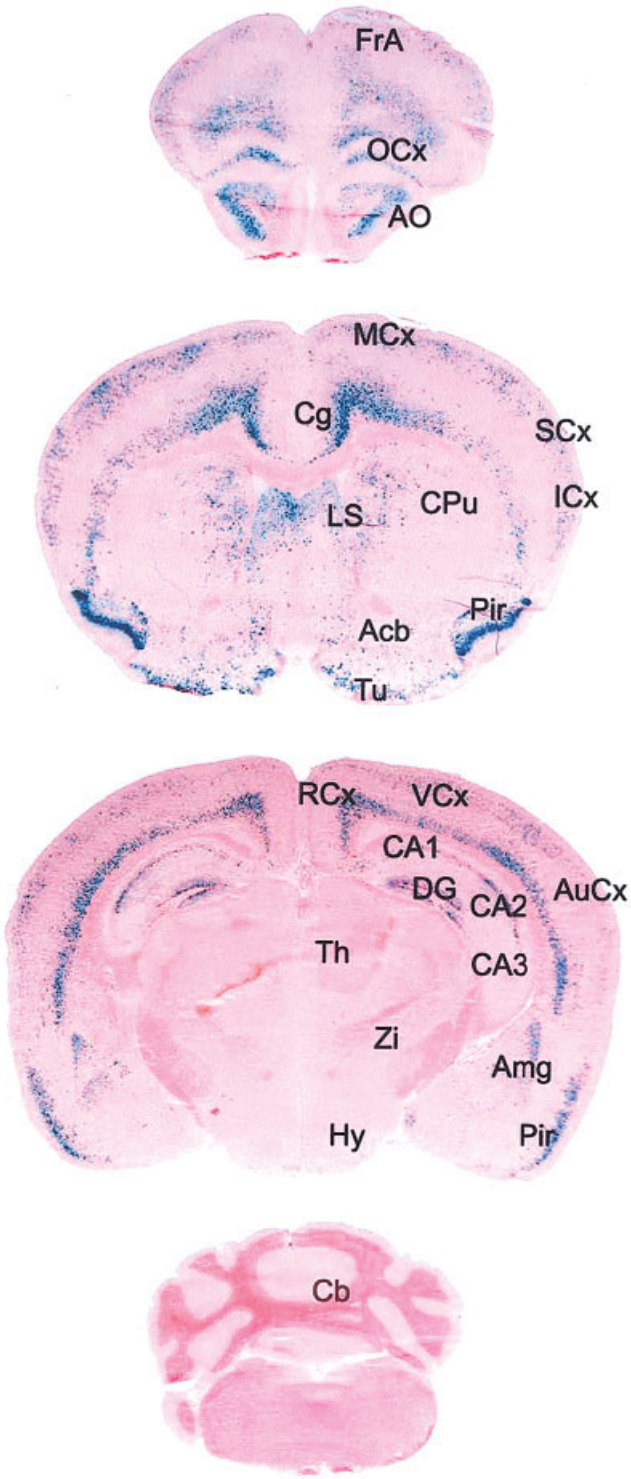


FIG. 2. Dox-dependent gene expression with the rtTA2 system. Double transgenic adult mice carrying the CaMKIIα promoter-rtTA2 (line 898) and tetO-lacZ transgenes were fed 6 mg/g dox for 11 days. β-Galactosidase activity was detected in forebrain structures, including nucleus accumbens (Acb), amygdala (Amg), anterior olfactory nuclei (AO), auditory cortex (AuCx), cornu ammonis regions 1,2,3 (CA 1,2,3), cingulate cortex (Cg), caudate putamen (CPu), dentate gyrus (DG), frontal association cortex (FrA), insular cortex (ICx), lateral septum (LS), motor cortex (MCx), orbital cortex (OCx), piriform cortex (Pir), retrosplenial cortex (RCx), somatosensory cortex (SCx), olfactory tubercles (Tu), visual cortex (VCx). There was no expression in midbrain or hindbrain structures such as the cerebellum (Cb), hypothalamus (Hy), thalamus (Th), zona incerta (Zi), or brain stem (Bs). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

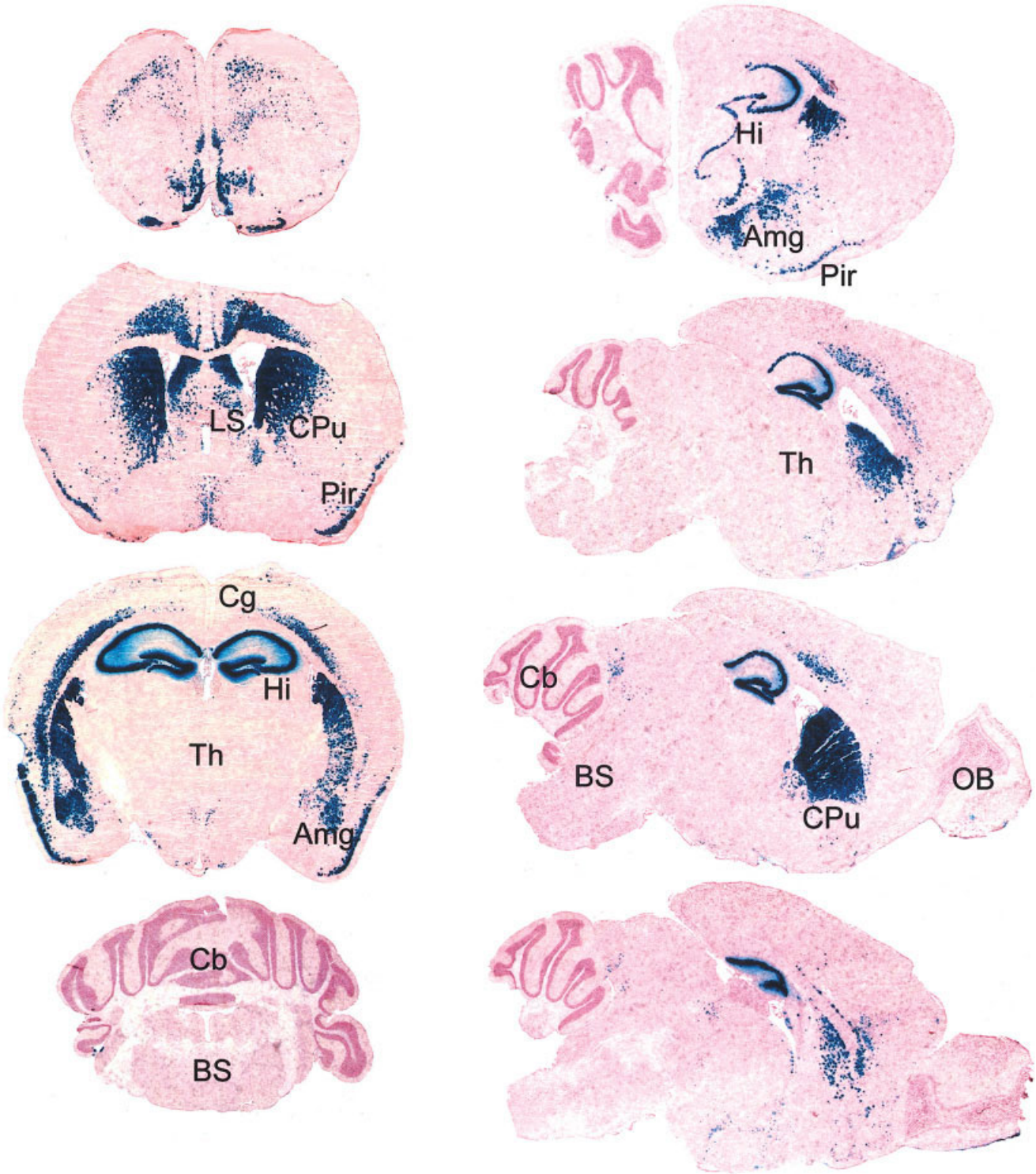
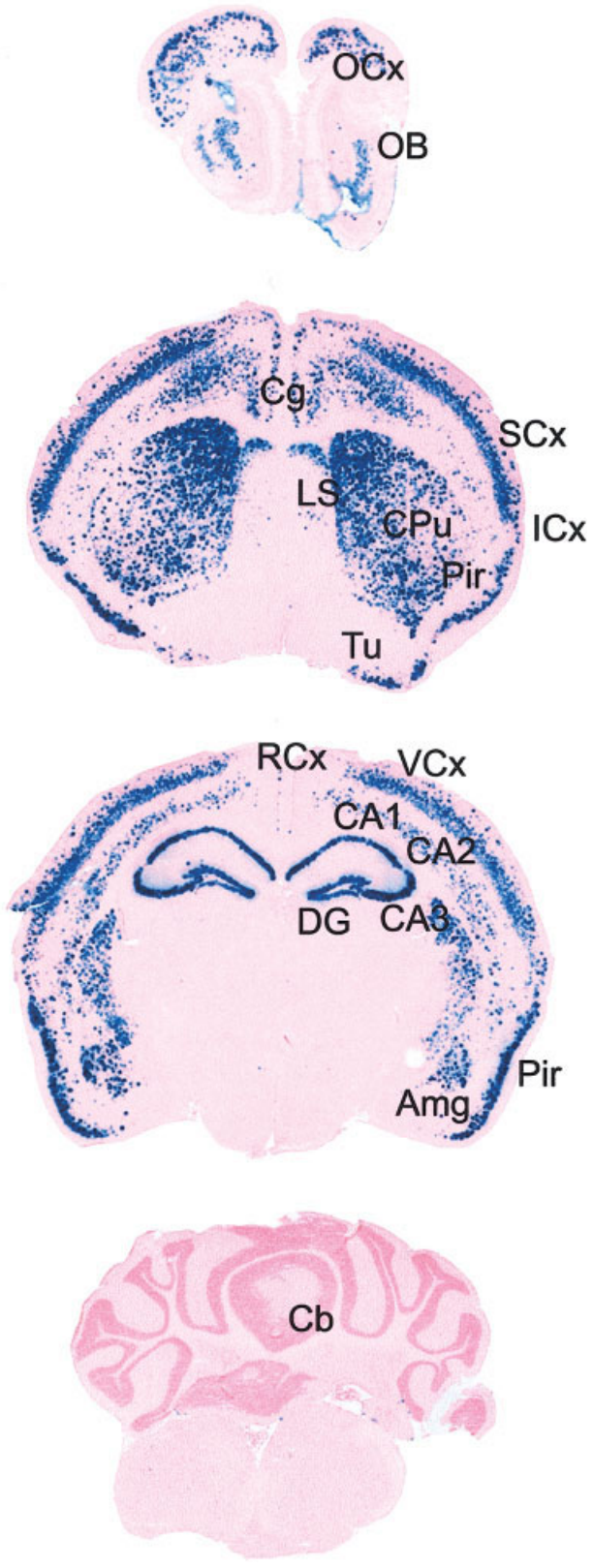


FIG. 3. Expression pattern in a bitetO promoter-lacZ reporter line (bitetO-lacZ line 1). Coronal (**a**) and sagittal (**b**) brain sections from adult double transgenic mice carrying CaMKIIα promoter-rtTA2 (line 898) and bitetO-lacZ transgene (line 1) fed dox (6 mg/g in food). β-Galactosidase expression is restricted to forebrain structures including the amygdala (Amg), hippocampus (Hi), cingulate cortex (Cg), caudate putamen (CPu), lateral septum (LS), and piriform cortex (Pir). There was no expression in the olfactory bulb (Ob), thalamus (Th), brainstem (Bs), or cerebellum (Cb).



dependent gene expression in the adult brain. With these mice, gene expression can be induced in a dose- and time-dependent fashion in neurons of most forebrain structures including cortex, hippocampus, amygdala, and striatum. Expression overall is consistent in several independent rtTA2-responsive lines whether carrying a tetO or bitetO promoter. The slight variations in the pattern and level of expression most likely result from different integration site, copy number, or postintegration modifications of the tetO-carrying transgene such as recombination or epigenetic changes (Mayford *et al.*, 1996; Robertson *et al.*, 2002). In this respect, the lower lacZ expression observed in the tetO-lacZ reporter line reflects a general reduction in transactivation efficiency that may result from extensive back-crossing of the line since its creation.

It should be noted that some properties of rtTA2 reported in vitro were not replicated in vivo. The rtTA2 factor was shown to require 10 times less dox than rtTA for full transactivation (0.1 versus 1 μg dox/ml) in stably transfected cells (Urlinger *et al.*, 2000). In vivo, however, we observed that both the rtTA and rtTA2 factors required a similar dose of dox to achieve comparable kinetics of gene induction. This discrepancy suggests that the sensitivity of rtTA and rtTA2 for dox may be similar in vivo (unlike in vitro) and, in turn, that rtTA2 is less effective in vivo than in vitro due to unknown mechanisms. In the future, the design of more efficient drugs may help enhance the efficacy of these expression systems. For instance, 4-epidoxycycline (4-ED), a hepatic metabolite of dox without antimicrobial activity or side effects on intestinal flora, was recently shown to be an efficient alternative to dox for regulating transgene expression in mice (Eger *et al.*, 2004). A new induction system using photolabile-caged dox was also developed for cell-specific delivery and local gene expression that may be useful in vivo (Cambridge *et al.*, 2004). Alternatively, more sensitive transactivators may be created in the future.

MATERIALS AND METHODS

Generation of CaMKIIα Promoter-rtTA2 Transgenic Mice

The rtTA2^{S}-M2 coding sequence was flanked with hybrid regulatory sequences and fused to an 8.5-kb fragment of the CaMKIIα gene. To create this construct, the rtTA2S-M2

FIG. 4. Expression pattern in a second bitetO promoter-lacZ reporter line (bitetO-lacZ line 2). **a:** Coronal brain sections from adult double transgenic mice carrying CaMKIIα promoter-rtTA2 (line 898) and bitetO-lacZ transgene (line 2) fed dox (6 mg/g in food). β-Galactosidase expression was observed in the amygdala (Amg), cornu ammonis regions 1,2,3 (CA 1,2,3), cingulate cortex (Cg), caudate putamen (CPu), dentate gyrus (DG), insular cortex (ICx), lateral septum (LS), olfactory bulb (OB), orbital cortex (OCx), piriform cortex (Pir), retrosplenial cortex (RCx), somatosensory cortex (SCx), olfactory tubercles (Tu), visual cortex (VCx). There was no expression in midbrain or hindbrain structures such as the cerebellum (Cb), hypothalamus, thalamus, or brain stem. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

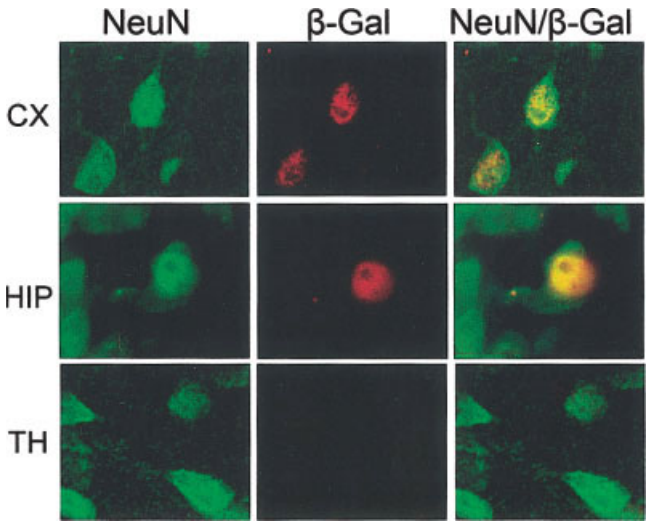


FIG. 5. Neuronal-specificity of gene expression with CaMKIIα promoter-rtTA2 mice. Colocalization of the neuron-specific marker NeuN (green, left panel) and β-galactosidase (red, middle panel) in neurons of cortex and hippocampus as indicated by the yellow signal (right panel) in double transgenic mice (line 898/tetO-lacZ). Neurons in thalamus do not express lacZ (no β-galactosidase signal). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

gene was first excised from the pUHD-rtTA2-MT2 plasmid with EcoR1 and BamH1 (Urlinger *et al.*, 2000) and blunted. This fragment was inserted into the EcoRV site of the pNN265 plasmid carrying 5′ and 3′ regulatory sequences with hybrid introns favoring transgene expression (Choi *et al.*, 1991) and a polyadenylation signal from SV40. This construct was removed by Not1 excision and inserted downstream of the CaMKIIα promoter at a Not1 site of the pMM403 plasmid (Mayford *et al.*, 1996). The full construct was excised from the vector with Sfi1, isolated from the vector backbone, and purified by electroelution followed by ethanol precipitation. DNA was microinjected in pronuclei of hybrid B6D2F1 eggs. Resulting founders were backcrossed to C57/Bl6J mice and animals from the F1 generation were crossed with mice heterozygous for tetO-LacZ (Mayford *et al.*, 1996) or bitetO-LacZ reporter gene. Animals carrying both transgenes and control littermates carrying no transgene or either one of the two transgenes were used (3–8 months old).

Mouse Housing

Mice were housed under constant temperature, humidity, and an inverted light/dark cycle of 12 h (dark 9:30 AM to 9:30 PM). Housing and maintenance conditions were according to guidelines established by the Federation of Swiss Cantonal Veterinary Officers.

Genotyping

Mice were marked by ear punching and genotyped by polymerase chain reaction (PCR) on tail DNA prepared with the HOTSHOT method (Truett *et al.*, 2000). For rtTA2, primers F1 (5′-TGCCTTTCTCTCCACAGGTGTCC-3′) and rtTA2-260R (5′-GAGAGCACAGCGGAATGAC-3′) were used. For lacZ, primers lacZ-s (5′-CCCATTACGGCT-AATCCGCCG-3′) and lacZ-as (5′-GCCTCCAGTACAGCG-CGGCTG-3′) were used. The PCR reaction contained 2.5 mM $MgCl_2$ and was run at 94°C for 30 s, 62°C for 40 s, and 72°C for 1 min for 35 cycles.

Doxycycline Administration

Dox (West-ward Pharmaceutical, Eatontown, NJ) was mixed in soft mouse chow (softened in sterile water, 50/50 w/v) or drinking solution (Sigma, St. Louis, MO; 5% sucrose). Medicated food was prepared daily and given at the beginning of the dark phase (morning) to minimize light exposure. Dox solutions were provided ad libitum in opaque bottles and renewed once (2 mg/ml and 0.05 μg/ml) or twice (3.5 mg/ml) a week.

β-Galactosidase Staining

Sixteen-μm fresh brain sections were fixed for 10 min in ice-cold 100 mM phosphate buffer, pH 7.2, containing 0.2% glutaraldehyde, rinsed three times, and incubated overnight at 37°C in 5 mM potassium ferrocyanide, 5 mM potassium ferrocyanate, 2 mM $MgCl_2$, 1 mM X-Gal, 0.01% sodium deoxycholate, and 0.02% NP40 in 100 mM phosphate buffer, pH 7.2. Sections were counterstained with acidified hematoxylin, dehydrated, then mounted with Neo-mount (Merck, Darmstadt, Germany) or DPX (Taab, UK).

RT-PCR

Total RNA was isolated with TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. Four μl Polyacryl Carrier (Research Center) was added to each sample to enhance RNA yield. Total RNA was treated with 3–4 units of RQ1 RNase free DNase (Promega, Madison, WI) in 1× RQ1 DNase Reaction Buffer (Promega) 2–3 times to remove potential DNA contamination. The reaction was terminated by adding 2 mM RQ1 DNase Stop Solution (Promega). Total RNA was purified by precipitation using sodium acetate and ethanol and stored at −80°C. Reverse transcription of mRNA to cDNA was conducted using the Enhanced Avian HS RT-PCR kit (Sigma), according to the manufacturer's instructions. Approximately 1 μg of total RNA was used in each reaction. The cDNA samples were then tested for the presence of lacZ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene used as a positive control, using lacZ forward (5′-CCCATTACGGCT-AATCCGCCG-3′) and reverse (5′-GCCTCCAGTACAGCG-CGGCTG-3′) primers, and GAPDH forward (5′-CACTGA-GCATCTCCCTCACA-3′) and reverse (5′-GTGGGTGCA-GCGAACTTTAT-3′) primers. The PCR reaction contained 2.5 mM (for lacZ) or 1.5 mM (for GAPDH) $MgCl_2$ and reacted at 94°C for 30 s, 62°C for 40 s, and 72°C for 1 min for 35 cycles (lacZ) or 94°C for 30 s, 56°C for 45 s, and 72°C for 45 s for 40 cycles (GAPDH). PCR products were run on 2.5% agarose gel in 0.5× TBE with ethidium bromide and visualized by UV.

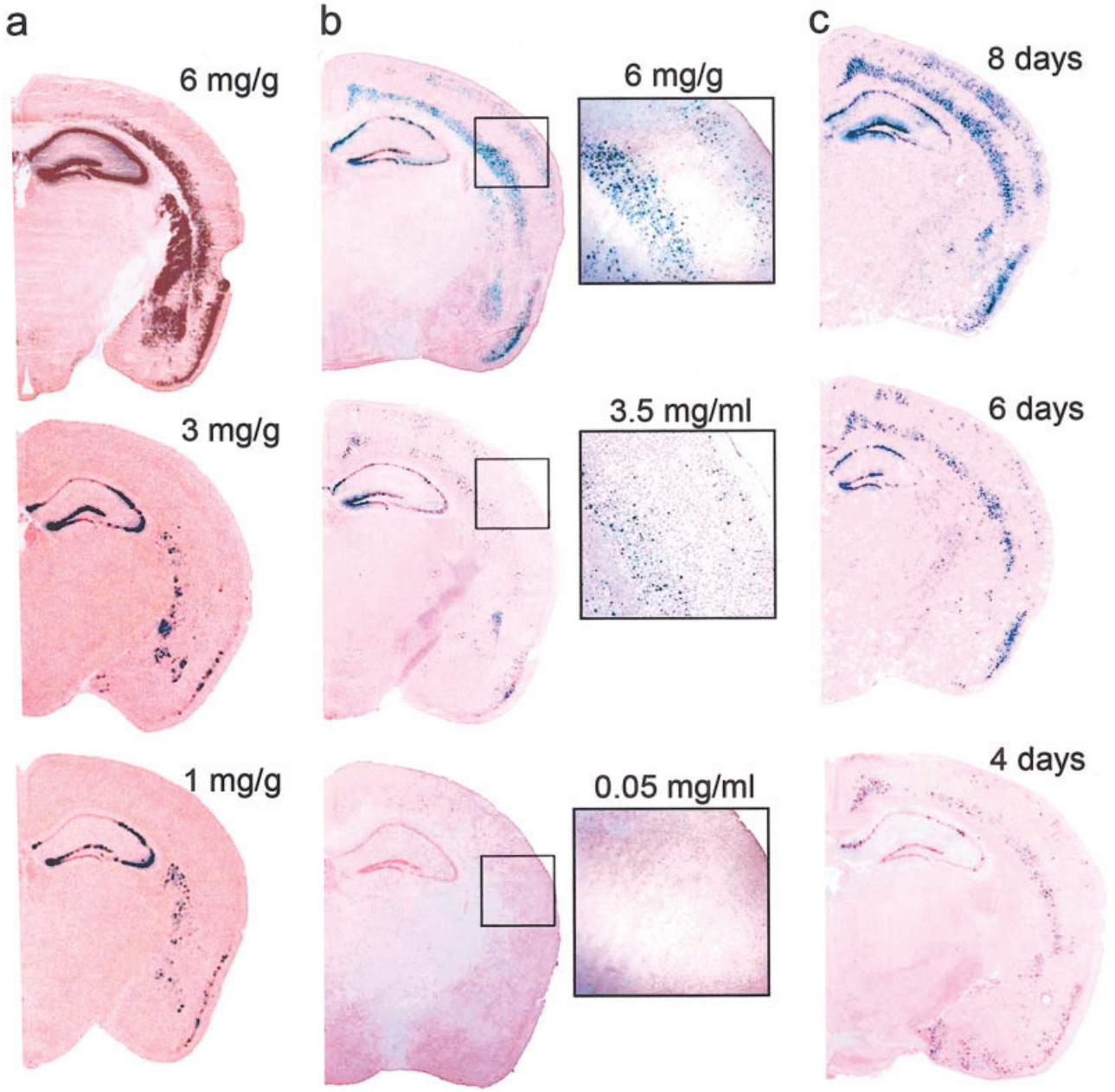


FIG. 6. Dose-dependent gene expression with the rtTA2 system using bitetO-lacZ (**a**) or tetO-lacZ line (**b**). **a:** Double transgenic mice (line 898/bitetO-lacZ line 1) received 6 mg/g, 3 mg/g, or 1 mg/g dox in food for 18–20 days. **b:** Double transgenic mice (line 898/tetO-LacZ) received 6 mg/g dox in food, or 3.5 mg/ml or 0.05 mg/ml dox in drinking water for 1 month. A long treatment was chosen to avoid interference between dox dosage and induction kinetic. The strongest expression was obtained with 6 mg/g dox in food, while no expression was induced at 0.05 mg/ml in drinking water. **c:** Time course of induction of dox-dependent gene expression. Double transgenic mice (line 898/tetO-lacZ) were fed 6 mg/g dox food for 2, 4, 6, or 8 days. A minimum of 4 days of dox treatment was required to induce lacZ expression and accumulation of β-galactosidase to a detectable level. The highest expression was detected after 8 days of dox treatment. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Immunohistochemistry

Mice were perfusion-fixed in 4% formaldehyde with 2.4 mg/ml sodium periodate and 0.1 M lysine and post-fixed in the same fixative for 1h at 4°C. The fixed brains were cut at 50 μm using a Leica VT1000s vibratome. Free-floating sections were washed with 0.3% Triton X-100 in phosphate-buffered saline (PBS), then three times in PBS alone. Sections were blocked in 4% dry milk in PBS at room temperature for 1 h and incubated in primary antibody for 2 days at 4°C and secondary antibody for 1.5 h. All antibodies were diluted in 1% BSA in PBS using the following dilutions: 1:200 mouse anti-NeuN

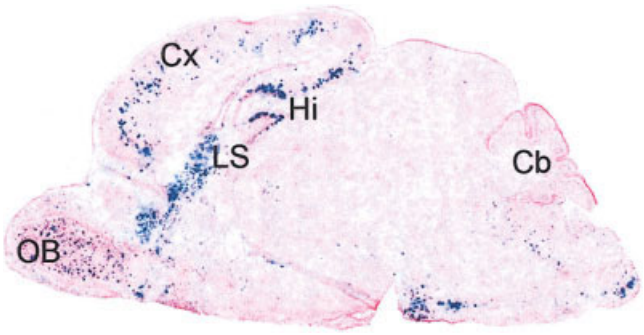


FIG. 7. β-Galactosidase activity in newborn pups (line 898/tetO-lacZ) after dox treatment of gestating dams. Forebrain structures showed β-galactosidase expression, including cortex (Cx), hippocampus (Hi), lateral septum (LS), and olfactory bulb (Ob). β-Galactosidase activity was quasi-absent from midbrain and cerebellum (Cb), but ectopic expression was detected in brain stem (Bs). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Abcam, Cambridge, UK), 1:300 rabbit anti-β-galactosidase (Molecular Probes, Eugene, OR), 1:500 FITC goat anti-mouse secondary antibody (Abcam), 1:500 Cy3 goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Images were digitized using a DMRE confocal microscope and a TCS SP2 scan head (Leica).

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