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Transgenic Animals with Inducible, Targeted Gene Expression in Brain

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ABSTRACT

Several inducible gene expression systems have been developed *in vitro* in recent years to overcome limitations with traditional transgenic mice. One of these, the tetracycline-regulated system, has been used successfully *in vivo*. Nevertheless, concerns remain about the ability of this system to direct high levels of transgene expression *in vivo* and to enable such expression to be turned on and off effectively. We report here the generation, using a modified tetracycline-regulated system under the control of the neuron-specific enolase promoter, of several lines of mice that direct transgene expression to specific brain regions, including the striatum, cerebellum, CA1 region of the hippocampus, or deep layers of cerebral neocortex. Transgene expression in these mice can be turned off completely with low doses of doxycycline (a tetracycline derivative) and driven to very high levels in the absence of doxycycline. We demonstrate this tissue-specific, inducible expression for three transgenes: those that encode luciferase (a reporter protein) or ΔFosB or the cAMP-response element binding protein (CREB) (two transcription factors). The various lines of transgenic mice demonstrate an inducible system that generates high levels of transgene expression in specific brain regions and represent novel and powerful tools with which to study the functioning of these (or potentially any other) genes in the brain.

The utility of transgenic mice for studying the function of a particular gene in the nervous system has been limited, because transgene expression typically occurs constitutively throughout development and in most tissues. As a result, any phenotypic abnormality observed in a particular brain region of adult mice, for example, could be caused by, or complicated by, abnormalities that occur any time during development or that exist in any other brain region or tissue of the adult animal. Inducible expression systems have been developed in recent years to overcome these limitations, and several have been shown to provide tight control of gene expression *in vitro* (e.g., Wyborski and Short, 1991; Gossen and Bujard, 1992; Wang *et al.*, 1994; Gossen *et al.*, 1995; Shockett *et al.*, 1995; No *et al.*, 1996). However, it has been difficult in many cases to demonstrate the ability of such systems to direct high levels of transgene expression *in vivo* and to turn such expression on and off effectively.

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J.C. and M.B.K contributed equally to this work.

Recently, inducible forebrain-specific expression of a mutant form of CaMKII has been achieved successfully with the use of the tetracycline-regulated system (Mayford *et al.*, 1996). This system involves two genes: one gene encodes tTA (a tetracycline-inhibitable transcription factor) and the other encodes the gene of interest under the control of the tTA-responsive TetOp promoter (Gossen and Bujard, 1992; Gossen *et al.*, 1995). In the 1996 Mayford report, tTA expression was placed under the control of a portion of the promoter of the *CaMKII* gene in an attempt to direct expression to certain forebrain regions. The resulting mice showed inducible and brain region-specific transgene expression, although the level of transgene expression achieved was not clear. In another study, high levels of transgene expression were obtained selectively in heart by use of the α myosin heavy chain promoter to drive tTA expression (Passman and Fishman, 1994). The latter finding raises the possibility that strong neuron-specific promoters could be used to direct particularly high levels of transgene expression in the brain.

The objective of the present study was 2-fold. First, we set out to determine whether such a strategy could be used to obtain inducible, region-specific expression of specific genes

ABBREVIATIONS: CaMKII, calcium/calmodulin-dependent protein kinase II; tTA, tetracycline transactivator; TetOp, tetracycline-responsive promoter; CREB, cAMP-responsive element binding protein; NSE, neuron-specific enolase; kb, kilobase; PCR, polymerase chain reaction; RT, reverse transcription.

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in brain. We focused on luciferase, a reporter protein, and two transcription factors that have been implicated in neural plasticity: ΔFosB (a member of the Fos family) and CREB (see Results and Discussion). Second, our goal was to achieve robust levels of transgene expression. To achieve the latter goal, we placed the *tTA* gene under the control of the NSE promoter. We used a 1.8 kb fragment of the promoter, which was shown previously to drive very high levels of expression of a reporter gene in brain (Forss-Petter *et al.*, 1990). We also used a modified form of the *tTA* gene to enhance levels of its expression.

We show here that the resulting bigenic mice support luciferase, ΔFosB, or CREB expression with striking region-specific patterns in brain and that such expression can be turned off completely or driven to very high levels by adding or removing doxycycline (a tetracycline derivative) in the drinking water. Preliminary reports of this work have appeared (Chen *et al.*, 1997b; Duman *et al.*, 1997a).

Materials and Methods

Construction of plasmids. Δ FosB cDNA in pcDEB vector under the control of the constitutive SR-α promoter was provided by Y. Nakabeppu (Kyushu University, Fukuoka, Japan) (Nakabeppu *et al.*, 1993). The ΔFosB cDNA was released from the pcDEBΔFosB by digestion with *Sal*I and *Bam*HI restriction endonucleases. The ends of the *Sal*I-*Bam*HI fragment was blunted with Klenow and subcloned into the blunted *Sal*I site of pTet-splice (Shockett *et al.*, 1995). The new plasmid was designated as pTetOp-ΔFosB, in which the ΔFosB was under the control of tetracycline-regulated promoter (TetOp). A 1.2-kb *Bam*HI-*Eco*RI fragment of the rat CREB cDNA (supplied by M. Montminy, Salk Institute, La Jolla, CA) was cloned into the blunted *Sal*I site of pTet-splice. The new plasmid was designated as pTetOp-CREB. An ~1.8-kb fragment of the NSE promoter in pNSE-LacZ (Forss-Petter *et al.*, 1990) was released by digestion with *Sac*I and *Hin*dIII, and subcloned into the ptTAk (Shockett *et al.*, 1995) in place of the TetOp-minimal cytomegalovirus promoter. The new plasmid was designated pNSE-tTA, in which the *Xho*I-*Hin*dIII fragment of the ptTAk was replaced by the SaCI-*Hin*dIII fragment of pNSE-LacZ. Construction of these plasmids, and their activity in cultured neural cell lines *in vitro*, have been reported recently (Chen *et al.*, 1997a).

Transgenic mice. DNA fragments (containing the promoter, open reading frame, SV40 intron, and poly(A)$^+$ signal) from pNSE-tTA, pTetOp-ΔFosB, and pTetOp-CREB were purified by electroelution and microinjected into the pronuclei of oocytes from SJL × C57BL6 mice. Tail DNA from resulting mice was isolated using a Tissue Amp DNA kit (Qiagen, Chatsworth, CA), and analyzed for the transgene by PCR, dot blotting, or Southern blotting (Sambrook *et al.*, 1989). Of these techniques, PCR was used for routine genotyping of the transgenic mice. The NSE-tTA transgene was detected by PCR with the primers NSE-F1: 23-mer, 5′ GTC CTC ATC CAT CAC TGC TTC CA 3′ and NSE-B1: 24-mer, 5′ CTA CCA GCT ATG TCT GTA GAG ACA 3′. The Δ*FosB* transgene was identified by PCR with the primers *FosB*F2: 20-mer, 5′ GAG TCT CAG TAC CTG TCT TC 3′ and FosBB2: 19-mer, 5′ GTC CAC TGG TGC TTG TGC T 3′. The TetOp-CREB transgene was detected by PCR with the primers CREB F1: 25-mer, 5′ CAG CCA TCA GTT ATT CAG TCT CCA C 3′ and CREB B1: 24-mer, 5′ GCT GCA TTG GTC ATG GTT AAT GTC 3′. The founder mice were crossbred with the ICR outbred mouse line to generate F1 mice. F2 homozygous transgenic mice were obtained by crossbreeding F1 siblings; homozygous genotype was confirmed by crossbreeding them with wild-type mice. Transgenic mice carrying both *TetOp-luciferase* and *TetOp-tTA* genes were provided by D. Schatz (Yale University, New Haven, CT) (Shockett *et al.*, 1995). To turn off transgene expression in mice carrying the *NSE-tTA* gene plus the *TetOp-luciferase*, *TetOp-ΔFosB*, or *TetOp-CREB* gene, the mice were fed with water containing doxycycline (Sigma Chemical, St. Louis, MO) and 5% sucrose. All of the transgenic mice used in this study were maintained in strict accordance with National Institutes of Health and institutional animal care guidelines.

Luciferase assay. Tissues from different organs or different brain regions were obtained by gross dissection. The tissues were homogenized in 500 μl of 1 × lysis buffer (according to manufacturer's specifications) using a sonicator or polytron. The homogenate was centrifuged for 5 min in a microcentrifuge. Ten microliters of the supernatant was used for measurement of luciferase activity in a luminometer by use of the luciferase reporter gene assay kit (Boehringer-Mannheim Biochemicals, Indianapolis, IN). Luciferase activity was normalized to total protein concentration.

Western blotting. One-dimensional Western blotting for ΔFosB was performed exactly as described previously (Chen *et al.*, 1997a), by using an anti-Fos-related antigen antibody (supplied by M. Iadarola, National Institutes of Health, Bethesda, MD) and chemiluminescence detection (Amersham, Arlington Heights, IL). Levels of ΔFosB immunoreactivity were quantified by measuring the optical density of specific bands using a Macintosh-based image analysis system with National Institutes of Health image software.

Immunohistochemistry. Immunohistochemical analysis of ΔFosB and of CREB was performed according to published procedures (Nibuya *et al.*, 1996; Hiroi *et al.*, 1997). Transgenic mice were perfused with 4% paraformaldehyde-phosphate-buffered saline. Brains were protected by 20% glycerol treatment overnight and cut into 40-μm sections with a microtome. Sections were labeled with a rabbit polyclonal anti-FosB antibody (1:5000; Santa Cruz Biochemicals, Santa Cruz, CA) or a rabbit polyclonal anti-CREB antibody (1:500, Upstate Biotechnology, Lake Placid, NY). Immunoreactivity was detected by diaminobenzidine staining by use of standard protocols (Nibuya *et al.*, 1996; Hiroi *et al.*, 1997).

RT-PCR. Total RNA was isolated from striatum of transgenic mice using the RNAqueous phenol-free total RNA isolation kit (Ambion, Austin, TX), and poly(A)$^+$ mRNA was isolated using the Oligotex mRNA mini kit (Qiagen, Chatsworth, CA). One microgram of poly(A)$^+$ mRNA was used as template for cDNA synthesis using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA). PCR was carried out according to standard protocols from Clontech. PCR primer pairs were designed to distinguish expression of the transgene from that of the endogenous gene. By use of a Δ*FosB* primer (FosBF2, see above) and an SV40 primer (SV40-B2: 24-mer, 5′ GTC AGC AGT AGC CTC ATC ATC ACT 3′), it was possible to detect expression of the Δ*Fos*B transgene, which contains both Δ*FosB* and SV40 sequences. Similarly, by use of a CREB primer (CREBF1, see above) and an SV40 primer (SV40-B2), it was possible to detect expression of the CREB transgene, which contains both CREB and SV40 sequences. By use of two *FosB* primers (m*FosB*F1: 23-mer, 5′ CCT TTG ACT CTT CTG TCT GAC CA 3′ and m*FosB*B1: 21-mer, 5′ AGC TAT CTT GGT CAC CCT GCA 3′) that were specific for the 3′ untranslated region of the endogenous gene, which is not included in the Δ*FosB* transgene, it was possible to detect endogenous FosB and ΔFosB mRNA.

***In situ* hybridization.** Frozen brains were cut into 16 mm sections. Sections were then subjected to *in situ* hybridization with a ^{35}S-labeled CREB riboprobe exactly according to published procedures (Nibuya *et al.*, 1996).

Results

Generation of transgenic mice with inducible, region-specific luciferase expression. The strategy we used to generate mice with inducible, targeted gene expression in brain, by use of the tetracycline-regulated system, is illustrated in Fig. 1A. The first step was to generate mice containing the *tTA* gene under the control of a 1.8-kb fragment

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of the NSE promoter, as described in Materials and Methods. We used a *tTA* gene with two modifications intended to enhance levels of tTA expression: 1) an SV40 intron was introduced into the 3′ untranslated region of the *tTA* gene to enhance stability of the encoded mRNA, and 2) the *tTA* gene contained mutations around the start AUG codon to enhance translation efficiency of the mRNA (see Discussion).

Twelve founder mice carrying the *NSE-tTA* gene were identified by PCR and Southern blotting. Four lines of these NSE-tTA mice were crossbred with a transgenic TetOp reporter mouse line, which carries two genes: *TetOp-luciferase* and *TetOp-tTA*. The *TetOp-tTA* gene had been engineered into this line as a way to provide higher levels of tTA expression (Shockett *et al.*, 1995). However, these bigenic reporter mice showed very low levels of luciferase expression in the brain and most other tissues even in the absence of tetracycline. Such low levels of expression were replicated in the present study, as shown in Fig. 1B.

In contrast, under such tetracycline-free conditions, the progeny from our crossbreeding, which carry the *NSE-tTA*,

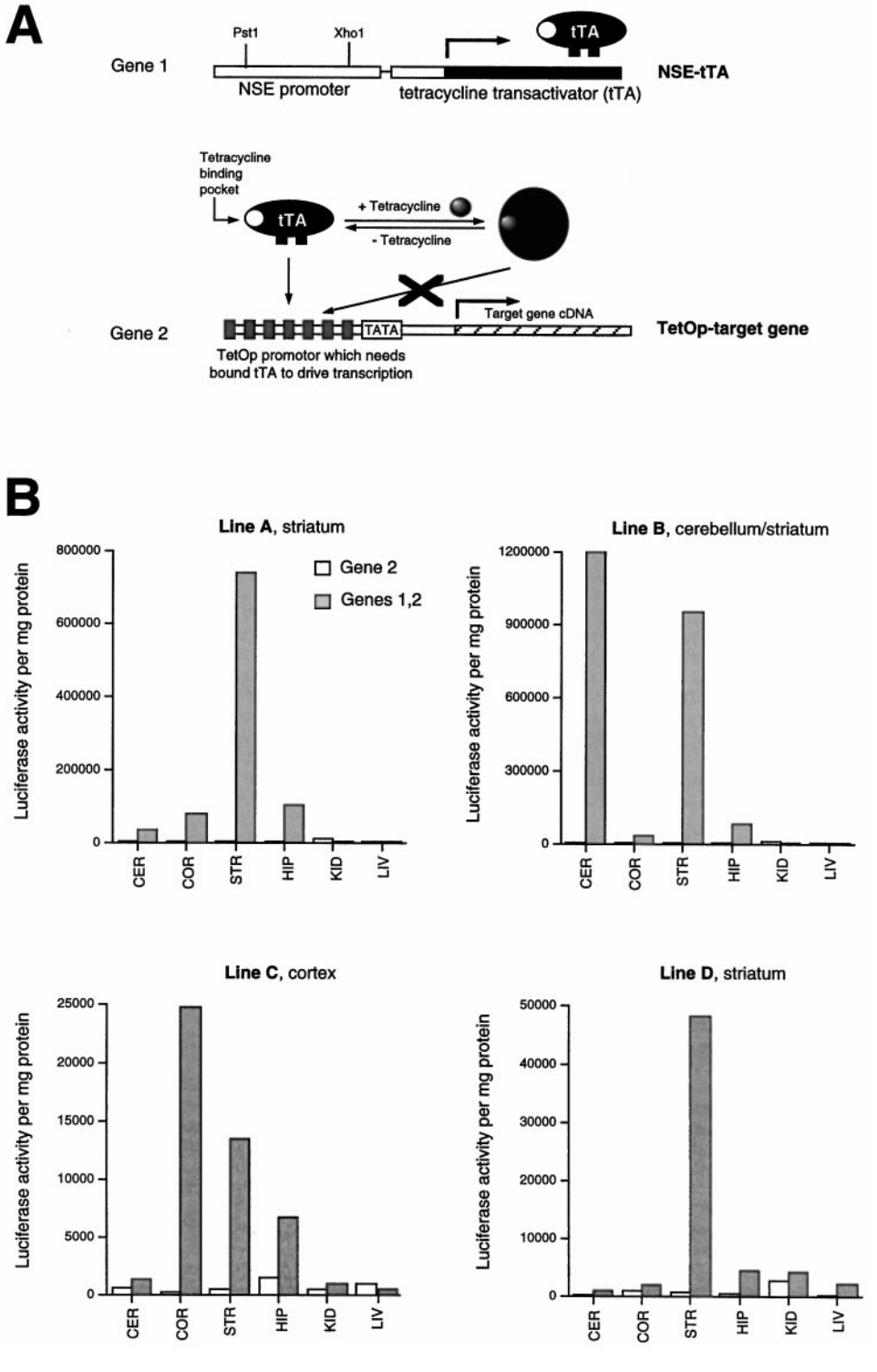

Fig. 1. A, Schematic diagram of a neuron-specific tetracycline-regulated gene expression system. Gene 1 encodes tTA under the control of the NSE promoter. Gene 2 encodes the gene of interest under the control of the tetracycline-responsive promoter, TetOp. B, Analysis of tissue-specific expression of luciferase in four lines of transgenic mice carrying the *NSE-tTA* gene (gene 1) plus the *TetOp-luciferase* and *TetOp-tTA* genes (gene 2). The *TetOp-tTA* gene was added as a positive autoregulatory feedback mechanism to enhance levels of tTA expression (Shockett *et al.*, 1995). Lines A and D showed highest level of luciferase expression in striatum, line B in cerebellum and striatum, and line C in cerebral cortex. Results are representative of the analysis of at least three animals in each treatment group.

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TetOp-luciferase, and *TetOp-tTA* genes, were found to express very high levels of luciferase in the brain, but not in peripheral tissues such as kidney and liver (Fig. 1B). Moreover, the various lines of NSE-tTA mice were found to direct different patterns of luciferase expression in the brain. Two lines (lines A and D) showed the highest levels of luciferase expression in striatum, one line (line B) in both cerebellum and striatum, and one line (line C) in cerebral cortex. These differences in expression patterns among the NSE-tTA mice are presumably due to the different insertion sites of the *NSE-tTA* gene.

One concern with the tetracycline-regulated system is the potential toxicity of the tTA protein: its transactivation domain is derived from the VP16 gene of the herpes simplex virus. However, we observed no detectable toxicity in our NSE-tTA mice. The mice yielded normal-size litters, with roughly equal numbers of mutants and wild-types, and the progeny seemed normal in all respects. Indeed, one useful feature of the NSE promoter is that its expression is turned on at relatively late stages of development (Forss-Petter *et al.*, 1990). These findings are consistent with earlier reports in cell culture, wherein no toxic effects (e.g., cell death or slowed cell growth) of tTA were observed in several neural cell lines transfected stably or transiently with the *NSE-tTA* or *TetOp-tTA* gene (see Chen *et al.*, 1997a).

Generation of transgenic mice with inducible, region-specific expression of ΔFosB. To test whether these NSE-tTA mice could be used as tools to direct tissue-specific expression of a gene of interest other than a reporter gene, we generated mice carrying the *TetOp-ΔFosB* gene (see Materials and Methods) and crossed them with two lines (lines A and B) of the NSE-tTA mice. ΔFosB, a truncated splice variant of the *FosB* gene, is a Fos family member transcription factor (Nakabeppu and Nathans, 1991). It is known to be induced in a region-specific manner in brain in response to several types of chronic (but not acute) perturbation, including drugs of abuse, antipsychotic drugs, antidepressant drugs, seizures, and lesions (see Hope *et al.*, 1994a, 1994b; Pennypacker *et al.*, 1994; Chen *et al.*, 1997a; Hiroi *et al.*, 1997; Mandelzys *et al.*, 1997). Recent work has provided direct evidence for an important functional role of this protein under some of these conditions (Hiroi *et al.*, 1997, 1998).

Bigenic NSE-tTA × TetOp-ΔFosB mice were found to express very high levels of ΔFosB, which migrates as two isoforms of 35 and 37 kD (see Chen *et al.*, 1997a), as analyzed by Western blotting (Fig. 2). Consistent with the patterns of luciferase expression stated above (Fig. 1B), line A NSE-tTA mice directed highly selective expression of ΔFosB to striatum (Fig. 2A), whereas line B directed expression to striatum and cerebellum (Fig. 2B). In contrast, mice containing only the *TetOp-ΔFosB* gene showed no detectable ΔFosB expression (Fig. 2A and B), consistent with the absence of significant leak expression from this transgene and with the very low levels of expression of the endogenous gene under control conditions (Hope *et al.*, 1994b; Hiroi *et al.*, 1997).

Importantly, treatment of mice with doxycycline, a tetracycline analog with higher penetration of the brain-blood barrier and 1000-fold higher affinity for tTA, completely shut off ΔFosB expression in the NSE-tTA × TetOp-ΔFosB mice (Fig. 2C). These findings highlight the tissue-specific and inducible nature of transgene expression supported by these bigenic animals.

Further information on ΔFosB expression in NSE-tTA × TetOp-ΔFosB mice was obtained by immunohistochemistry,

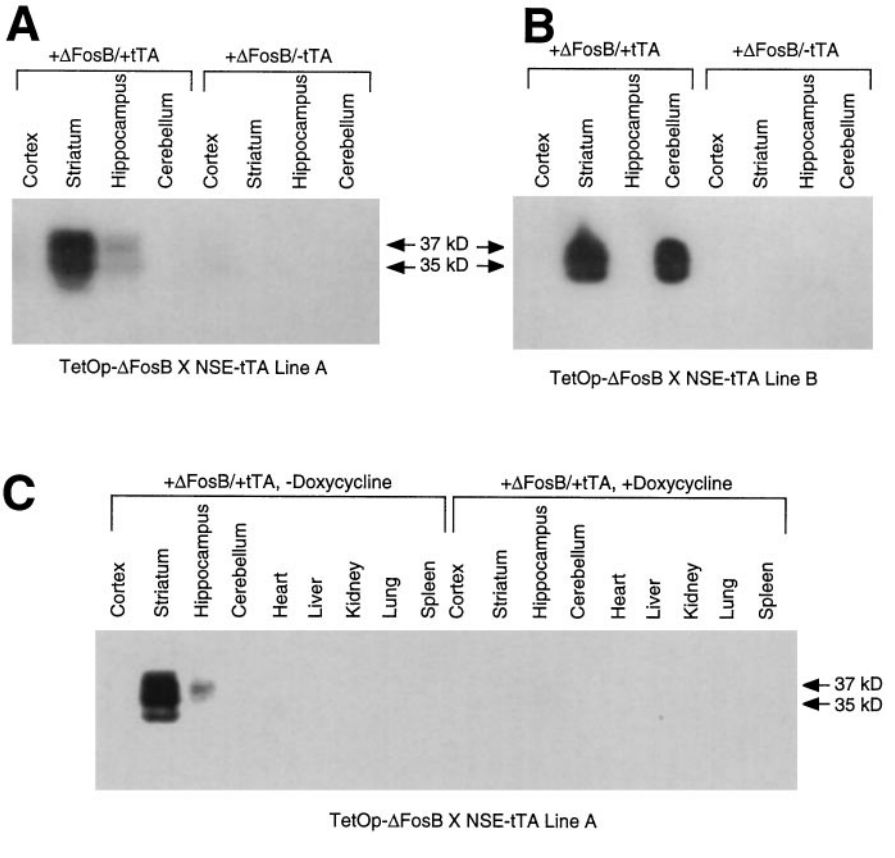

Fig. 2. Tissue-specific expression of ΔFosB in NSE-tTA × TetOp-ΔFosB transgenic mice detected by Western blotting. A, NSE-tTA (line A) × TetOp-ΔFosB mice. Expression of ΔFosB, which migrates at 35–37 kD (Chen *et al.*, 1997; Hiroi *et al.*, 1997), was highly enriched in striatum of the bigenic mice (+ΔFosB/+tTA), but not in mice carrying only the *TetOp-ΔFosB* gene (+ΔFosB/−tTA). B, NSE-tTA (line B) × TetOp-ΔFosB mice. ΔFosB was detected in cerebellum and striatum of the bigenic mice. C. NSE-tTA (line A) × TetOp-ΔFosB mice. ΔFosB expression in bigenic mice, which was highly enriched in striatum and not seen in several peripheral tissues, was turned off completely by including 200 μg/ml of doxycycline in the drinking water. Results are representative of the analysis of at least three animals in each treatment group.

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using a specific anti-FosB antibody (see Materials and Methods). As shown in Fig. 3A, line A NSE-tTA mice directed selective and high levels of ΔFosB expression in striatum, with highest levels seen in dorsal-medial aspects of this structure. ΔFosB-like immunoreactivity was largely nuclear, as would be expected given its role as a transcription factor. Consistent with the Western blot data, lower levels of ΔFosB expression were evident in cerebral cortex (where expression was enriched in deep layers) and in the hippocampus (where expression was highly specific for the CA1 subfield) (Fig. 3A). The same expression patterns were observed in the progeny from crossbreedings between line A NSE-tTA mice and two other TetOp-ΔFosB lines (data not shown), although these bigenic mice showed different overall levels of ΔFosB expression—one higher and one lower than that shown in Fig. 3A—presumably based on their insertion sites in the genome.

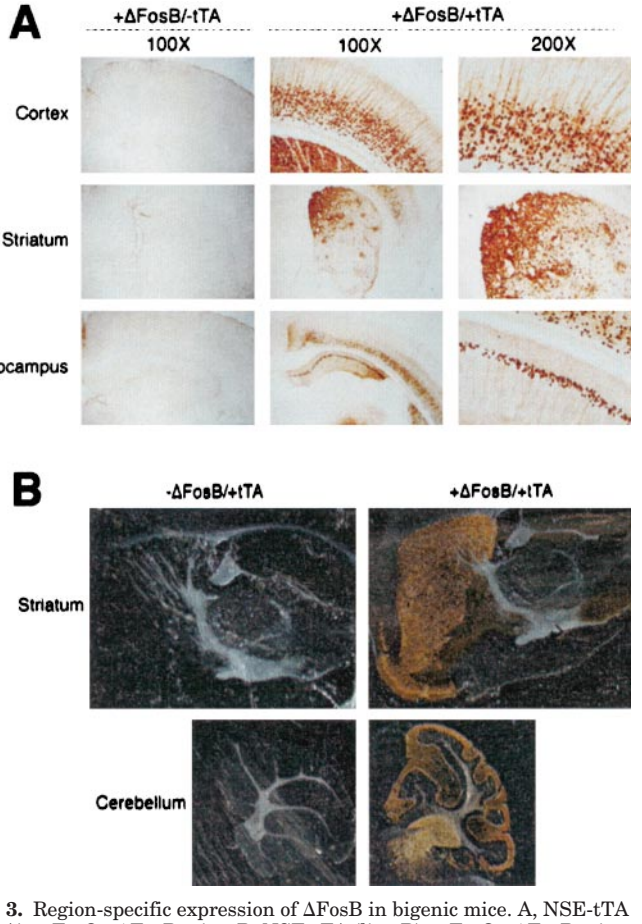

Fig. 3. Region-specific expression of ΔFosB in bigenic mice. A, NSE-tTA (line A) × TetOp-ΔFosB mice. B, NSE-tTA (line B) × TetOp-ΔFosB mice. +ΔFosB/−tTA, mice carrying only TetOp-ΔFosB; −Δ FosB/+tTA, mice carrying only NSE-tTA; +ΔFosB/+tTA, bigenic mice carrying both NSE-tTA and TetOp-ΔFosB. ΔFosB was detected by immunohistochemistry with an anti-FosB antibody as described in Materials and Methods. A, ΔFosB immunoreactivity in light field. *Top row*, high levels of Δ*Fos*B expression in dorsal-medial aspects of striatum. *Middle row*, lower levels of ΔFosB expression in deep layers of cerebral neocortex. *Bottom row*, ΔFosB expression that was highly restricted to the CA1 region of hippocampus, apparently within pyramidal neurons. B, ΔFosB immunoreactivity in dark field (ΔFosB immunoreactivity appears as brown staining on a black and white background). *Top*, high levels of ΔFosB expression selectively throughout dorsal and ventral striatum. *Bottom*, high levels of ΔFosB expression in cerebellum, which on higher magnification was localized to Purkinje cells (data not shown). Results are representative of the analysis of at least three animals in each treatment group.

In contrast to line A mice, progeny of line B NSE-tTA mice, when crossed with any of the three TetOp-ΔFosB mouse lines, showed high levels of ΔFosB expression in both striatum and cerebellum (Fig. 3B). These findings are consistent with Western blot analysis of these animals (see Fig. 2B). In the line B-derived mice, unlike the line A-derived mice, expression of ΔFosB was not enriched in dorsal-medial aspects of the striatum; rather, high levels of expression were seen throughout the dorsal-ventral and medial-lateral aspects of this brain region. Also in these line B-derived mice, cerebellar expression was highly enriched in Purkinje cells. Together, these results indicate that the NSE-tTA mice determine the pattern of transgene expression, whereas the TetOp-ΔFosB mice can influence the level of transgene expression.

To confirm that the increase in ΔFosB immunoreactivity in the NSE-tTA × TetOp-ΔFosB bigenic mice was due to induction of the Δ*FosB* transgene and not induction of the endogenous *FosB* gene, we analyzed mRNA from striatum of the bigenic mice by RT-PCR. Using a primer pair (*FosB*F2-SV40B2, see Materials and Methods) specific for Δ*FosB* and SV40 sequences contained within the Δ*FosB* transgene, we demonstrated robust expression of the transgene in bigenic mice carrying both the *NSE-tTA* and *TetOp-ΔFosB* genes, but not in transgenic mice carrying only the *NSE-tTA* gene (Fig. 4A). In contrast, expression of the endogenous *FosB* gene, detected by a primer pair (m*FosB*B1 and m*FosB*F1) specific for the 3′ untranslated region of FosB and ΔFosB mRNA (and

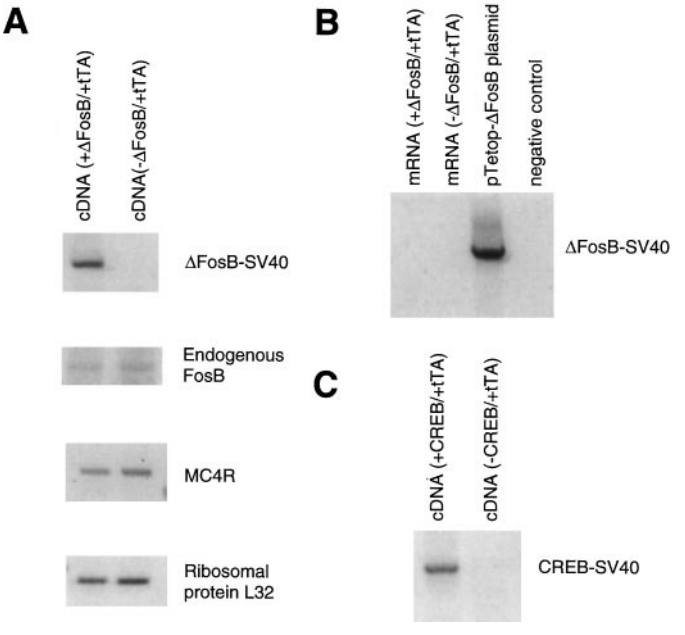

Fig. 4. Analysis of mRNA from striatum of transgenic mice by RT-PCR. poly(A)$^+$ mRNA from five transgenic mice in each group was pooled and used as template for synthesis of cDNA by reverse transcription. The cDNA was amplified by PCR with specific primer pairs (see Material and Methods). A, mRNA samples from bigenic mice carrying both the *NSE-tTA* and *TetOp-ΔFosB* genes, and from transgenic mice carrying only the *NSE-tTA* gene, were analyzed by RT-PCR with primers specific for the Δ*FosB* transgene or for endogenous FosB/ΔFosB, melanocortin-4 receptor, or ribosomal protein L32. B, mRNA samples from the transgenic mice were analyzed by regular PCR to confirm the lack of DNA contamination in the mRNA samples. The plasmid containing TetOp-ΔFosB was used as a positive control and water was used as a negative control for the PCR. C, mRNA samples from bigenic mice carrying both the *NSE-tTA* and *TetOpCREB* genes, and from transgenic mice carrying only the *NSE-tTA* gene, were analyzed by RT-PCR with primers specific for the CREB transgene or endogenous CREB.

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not present in the Δ*FosB* transgene) was similar between the NSE-tTA mice versus the NSE-tTA × TetOp-ΔFosB mice. Two other genes, those for ribosomal protein L32 and melanocortin-4 receptor, which were used as internal controls, also showed similar expression levels. To confirm that there was no contamination of the striatal mRNA samples with genomic DNA, regular PCR reactions were performed on these samples. No signals were detected in the striatal mRNA samples (Fig. 4B), which indicates the lack of DNA contamination. Together, these results indicate that the increase in ΔFosB immunoreactivity seen in the bigenic mice is due to the induction of the Δ*FosB* transgene.

Generation of transgenic mice with inducible, region-specific expression of CREB

Further evidence of the utility of the NSE-tTA mice in driving expression of a transgene of interest came from crossbreeding line A NSE-tTA mice with mice containing the *TetOp-CREB* gene (as described in Materials and Methods). CREB is a highly regulated transcription factor that has been implicated as an important mediator of many forms of neural plasticity, including learning and memory (see Mayford *et al.*, 1995; Yin and Tully, 1996; Kornhauser and Greenberg, 1997) and the long-term actions of drugs of abuse and antidepressant treatments (Hyman, 1996; Duman *et al.*, 1997b; Nestler and Aghajanian, 1997).

Fig. 5 shows that the line A NSE-tTA × TetOp-CREB mice support expression of CREB immunoreactivity that is enriched in dorsal-medial aspects of striatum. CREB immunoreactivity showed a clear nuclear localization, as would be expected for this transcription factor. A similar pattern of expression was seen for CREB mRNA as measured by *in situ* hybridization (data not shown). Note that the pattern of CREB expression in these mice is very similar to that seen for Δ*Fos*B in the line A mice (see Fig. 3A). This further supports the view, stated earlier, that the NSE-tTA mice determine the cell type-specificity of transgene expression. The increase in CREB immunoreactivity as detected by immunostaining, or in CREB mRNA level as detected by *in situ* hybridization, seems to be due to the induction of the CREB transgene. Thus, as shown in Fig. 4C, a primer pair (CREBF1-SV40B2) specific for CREB and SV40 sequences contained within the CREB transgene revealed strong expression of the transgene in bigenic mice carrying both the *NSE-tTA* and *TetOp-CREB* genes, but not in transgenic mice carrying only the *NSE-tTA* gene.

Control of transgenic expression with low doses of doxycycline

One of the major perceived drawbacks of the tetracycline-regulated expression system is the very high doses of doxycycline that have been used. Several groups have used more than 2 mg/ml of doxycycline in the drinking water to turn expression of the targeted gene off (Mayford *et al.*, 1996; Kistner *et al.*, 1996). However, these earlier studies did not assess the dose dependence of doxycycline action.

Because the NSE-tTA mice offer the advantage of high levels of transgene expression, we performed a dose-response study of doxycycline, first in the NSE-tTA × TetOp-luciferase × TetOp-tTA mice. It was found that 25 μg/ml of doxycycline in the drinking water turned luciferase expression off as effectively as any higher dose (Fig. 6). Even much lower doses of doxycycline, such as 2.5 and 0.25 μg/ml, substantially, albeit partially, turned expression off. These findings demonstrate that the level of transgene expression is adjustable *in vivo* by use of the tetracycline-regulated sys-

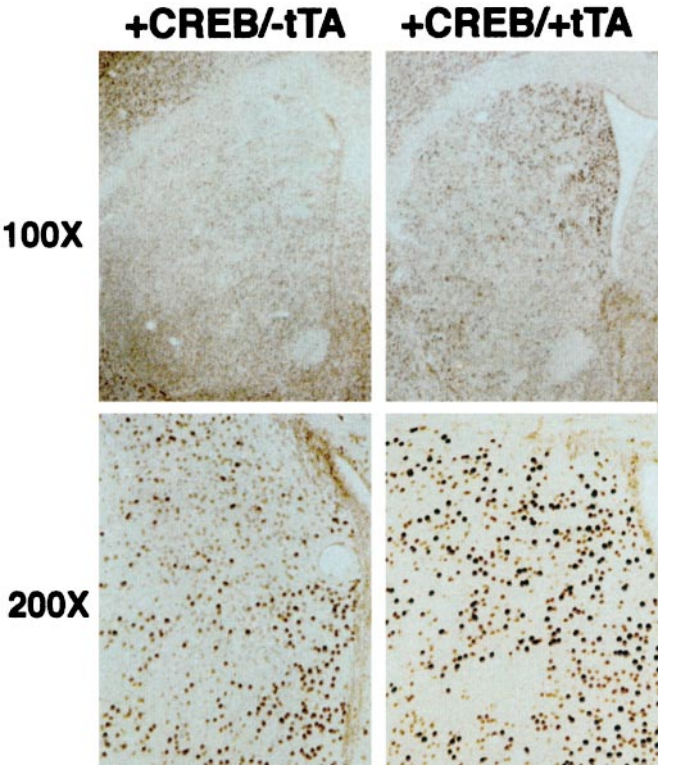

Fig. 5. Region-specific expression of CREB in bigenic mice. NSE-tTA (line A) mice were crossbred with TetOp-CREB mice. +CREB/−tTA, mice carrying only TetOp-CREB; +CREB/+tTA, bigenic mice containing both NSE-tTA and TetOp-CREB. CREB was detected by immunohistochemistry with an anti-CREB antibody as described in Materials and Methods. The +CREB/−tTA mice showed low, but widespread, levels of CREB expression that were indistinguishable from that seen in wild-type mice (data not shown). The +CREB/+tTA mice showed induction of CREB that was selective in dorsal-medial aspects of striatum. *Top*, low magnification; *Bottom*, higher magnification. Results are representative of the analysis of at least three animals in each treatment group.

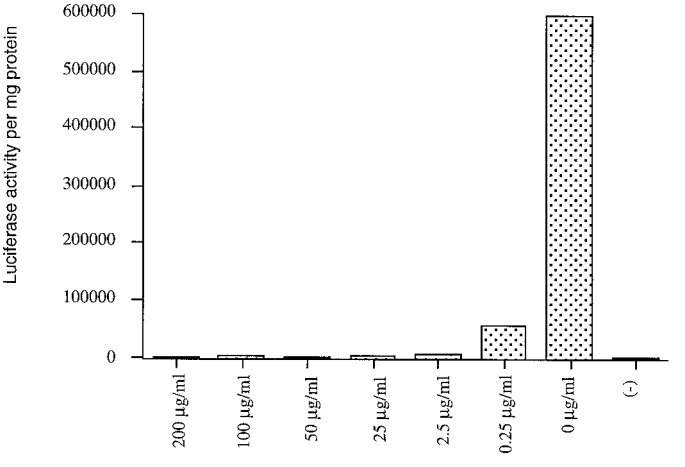

Fig. 6. Dose-response of luciferase expression in NSE-tTA (line A) × TetOp-luciferase × TetOp-tTA mice. The graph shows luciferase activity in striatum of trigenic mice, which had been exposed to drinking water containing various concentrations of doxycycline (from 0 to 200 μg/ml) for 3 weeks. −, control mouse containing the *TetOp-luciferase* and *TetOp-tTA* genes only.

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tem. Similar dose-response data were obtained for ΔFosB in NSE-tTA × TetOp-ΔFosB mice; for example, Fig. 2C shows complete blockade of ΔFosB expression at the 200 μg/ml dose of doxycycline. Such low doses of doxycycline are highly unlikely to produce any deleterious effects, because they result in blood levels far below those that are used clinically (e.g., the 200 μg/ml dose yields blood levels of <500 ng/ml, the lower limit of detection of the drug in clinical assays).

The use of lower doses of doxycycline is critical for more rapid induction of the transgene of interest. For example, it was found that bigenic mice expressing luciferase, which had been maintained on 2 mg/ml of doxycycline, showed only 10–15% induction of luciferase (compared with maximal levels of induction seen in the absence of doxycycline) after an 8 week washout period (Fig. 7A). In contrast, when mice were maintained on 50 μg/ml of doxycycline, luciferase expression was >50% of maximal levels within 2 weeks (Fig. 7B). Even more rapid induction was observed in mice maintained on 25 μg/ml of doxycycline. A similar time course of transgene expression was seen in the NSE-tTA × TetOp-ΔFosB mice. In fact, levels of ΔFosB attained in the brains of these mice after 2 weeks off doxycycline were comparable with levels of ΔFosB induced by chronic (2 weeks) administration of cocaine (data not shown). These data therefore establish that the tetracycline system can be used to obtain physiological induction of a transgene: physiological with respect to both the level and the time course of transgene expression.

Discussion

The major finding of this study is that NSE-tTA transgenic mice can be used as tools to direct high levels of inducible transgene expression to specific brain regions, including the striatum, cerebellum, CA1 region of hippocampus, and deep layers of neocortex. We demonstrate that genes encoding three different target proteins (luciferase, ΔFos, or CREB) under the control of the TetOp promoter were expressed in the same brain regions by any given line of the NSE-tTA mice. This finding suggests that the transgenic mouse line that carries the *tTA* gene under the control of some tissue-specific promoter determines the expression pattern of target genes under the control of the TetOp promoter.

The forebrain-specific CaMKII promoter has been used to direct tissue-specific expression of the *CaMKII* mutant and *Cre* genes (Mayford *et al.*, 1996; Tsien *et al.*, 1996). The expression patterns determined by the CaMKII-tTA mice are different from those determined by the NSE-tTA mice. Therefore, an important goal of future research is to develop more tTA-expressing mice, in which the *tTA* gene is under the control of various tissue-specific promoters to direct targeted gene expression to increasingly discrete and anatomically defined brain regions. Ultimately, a transgenic mouse bank containing different tissue-specific tTA transgenic mice could be very useful for inducible, tissue-specific expression of numerous target genes of interest.

A major distinctive feature of the NSE-tTA mice described in this study is that they support very high levels of transgene expression. Thus, this is the first case where inducible expression of a transgene in brain is demonstrated by Western blotting and immunohistochemistry. Indeed, the maximal level of ΔFosB induction that can be achieved with this system far exceeds levels of the protein induced in brain by various psychotropic drugs or other treatments. The high levels of transgene expression supported by our bigenic mice seems to have at least three causative factors. First, an SV40 intron was introduced into the 3′ untranslated region (Shockett *et al.*, 1995) [instead of the 5′ untranslated region (Mayford *et al.*, 1996)] of both the *tTA* gene and the target gene. 3′

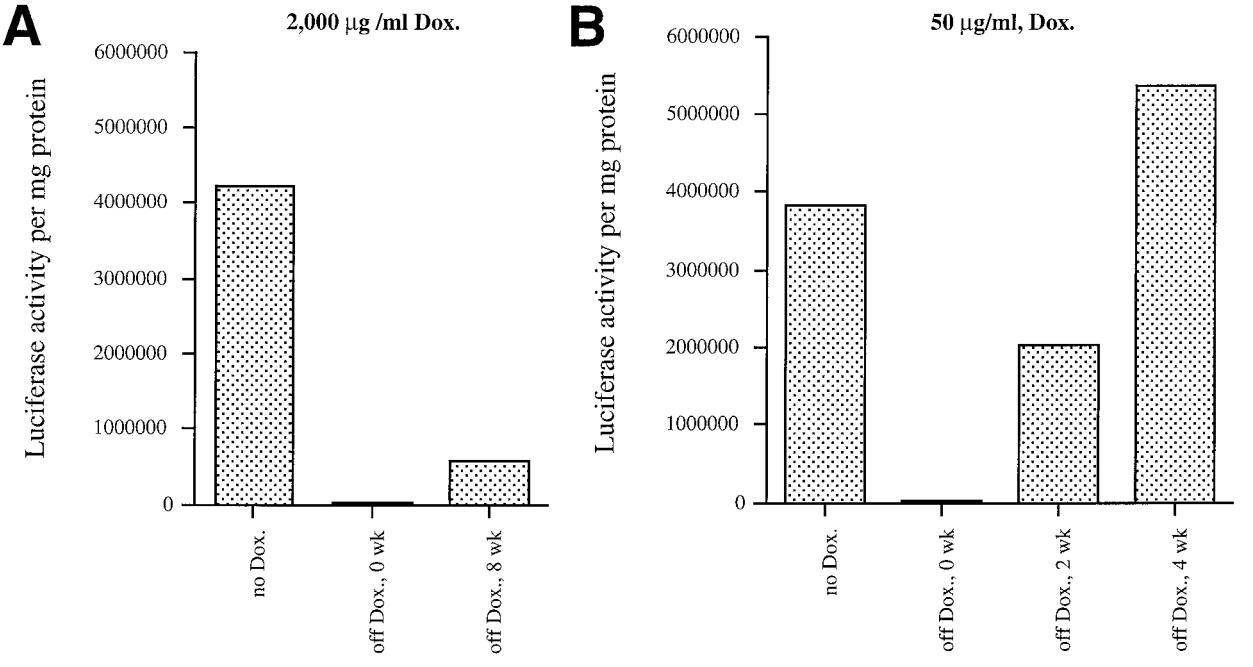

Fig. 7. Induction of luciferase expression in NSE-tTA (line A) × TetOp-luciferase × TetOp-tTA mice. Trigenic mice were exposed to drinking water containing 2,000 (A) or 50 (B) μg/ml of doxycycline in the drinking water from conception through adulthood. The graph shows luciferase activity in striatum of such adult mice from which doxycycline was then removed for the indicated time period (*off Dox, 0–8 wk*). Mice that were never exposed to doxycycline (i.e., in which gene expression had always been on) are shown for comparison (*no Dox*). Results are representative of the analysis of at least three animals in each treatment group.

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Introns have been shown to result in much higher levels of mRNA accumulation, presumably because of increased polyadenylation efficiency (Wassarman and Steitz, 1993; Gunderson *et al.*, 1997) or transport efficiency from the nucleus to the cytoplasm (Pante *et al.*, 1997). Second, the *tTA* gene used in the present study contains mutations around the start AUG codon to better match a Kozak consensus sequence, which can enhance translation efficiency of the mRNA (Kozak, 1984). Third, the NSE promoter is a strong promoter, which has been shown to drive high levels of expression of the *LacZ* reporter gene (Forss-Petter *et al.*, 1990) and the *bcl-2* gene (Farlie *et al.*, 1995) in neuronal tissues.

An important consideration in the generation of additional tTA-expressing mice will be the relative strength of the other neuron-specific promoters used to drive the *tTA* gene. Many promoters that direct highly restricted patterns of expression (e.g., those controlling specific receptor subtypes) may be weaker than the NSE promoter based on the much lower levels of expression of the encoded proteins. A lower level of tTA expression, and hence of target gene expression, may be encountered if such promoters are used. One potential solution is to include a third gene, *TetOp-tTA*, in the system (Shockett *et al.*, 1995). In this case, it is possible that a low level of tTA expression driven by a weak, but highly tissue-specific, promoter could trigger an autoregulatory positive feedback loop in the *TetOp-tTA* gene so that a higher level of tTA would be expressed in the specific tissue. Our results with such a three-gene regulation system (*NSE-tTA* × *TetOp-luciferase* × *TetOp-tTA*) show very high and tissue-specific levels of luciferase expression in the absence of tetracycline, but virtually no expression in the presence of tetracycline (Fig. 1B), which suggests that a three-gene regulation system is capable of tightly regulated transgene expression.

One major problem associated with the tetracycline-regulated system is the slow induction of transgene expression upon removal of standard doses of doxycycline (e.g., 2 mg/ml) from the drinking water (e.g., Fig. 7A). This has been a disappointing feature of the technology. We attempted to overcome this problem by dramatically reducing the dose of doxycycline used. Surprisingly, it was found that almost a one hundred-fold lower dose of doxycycline (25 μg/ml) was equally effective at turning transgene expression off as the higher doses (see Fig. 6). Use of such lower doses of doxycycline is critical, because it markedly shortened the induction time of transgene expression (Fig. 7B). The more rapid induction of transgene expression seen with the lower doses of doxycycline is presumably caused by the shorter period of time needed for the drug to be cleared from the animals. Additional advantages of the reduction in doxycycline dose are a decrease in potential nonspecific side effects of higher doses (2 mg/ml) and a greatly reduced cost of doxycycline.

The tTA system used in the present study is just one of several approaches that are being explored to obtain inducible and tissue-specific expression in transgenic mice (see Gingrich and Roder, 1998). One modification under investigation is the use of a mutated tTA protein, rtTA, which is activated, not inhibited, by tetracycline (see Freundlieb *et al.*, 1997). This approach holds promise for more rapid transgene induction, but has not yet been reported to work *in vivo*. Other inducible systems are under investigation, but the low induction ratios obtained with the progesterone-inducible system (Wang *et al.*, 1994) and the requirement for three transgenes in the ecdysone-inducible system (No *et al.*, 1996) represent current limitations that require further refinements.

In the meantime, the results of the present study establish the feasibility of obtaining high yet adjustable levels of tissue-specific, inducible gene expression in transgenic mice with the tTA system. Findings with the TetOp-luciferase, TetOp-ΔFosB, and TetOp-CREB mice indicate that the NSE-tTA mice are largely responsible for directing the pattern of transgene expression, regardless of the transgene involved. In addition, the findings show that the various lines of NSE-tTA mice can be used as novel tools to obtain tissue-specific and inducible expression of potentially any transgene placed under the control of the TetOp promoter. These mice represent powerful tools that will enable detailed studies of the function of individual genes in the adult brain.

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