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Improvement and establishment of the tTA-dependent inducible system in the mouse brain

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Heidelberg, September, 2001

Meinem Vater, dem Helden

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Summary

A genetic regulatory system employing the tetracycline-controlled transactivator (tTA) that can induce individual gene expression reversibly in a temporal and spatial manner could permit a more precise analysis of gene functions on animal physiology and behaviour. In this study we have described the optimization of the tTA coding sequence (humanized tTA, htTA) for efficient expression in the mouse and the establishment of the htTA-dependent inducible system by conditional means in transgenic mice. The coding sequence of the htTA was altered concerning mammalian codon usage, putative splicing signals, CG dinucleotide content, and toxicity of the transactivation domain VP16. These sequence changes on tTA resulted in the three-fold increase of the inducible activity in transiently transfected HeLa cells, compared to the prokaryotic one. Activity of the htTA was efficiently suppressed by addition of doxycycline.

To express the htTA in a neuron- and region-specific manner in mouse brain, the region-specific promoter of the high-affinity kainate receptor subunit KA1 was chosen. By using KA1 bacterial artificial chromosomes (BAC), it was expected that expression of htTA under the control of the KA1 promoter is directed to the areas expressing KA1 itself, namely in CA3 and dentate gyrus (DG) of the adult hippocampus. The functional activity of the htTA, however, was not observed in KA1-htTA transgenic mice, in spite of expression of the htTA mRNA as determined by RT-PCR. The htTA gene under the KA1 promoter appears to be expressed too low to induce tTA responsive genes (e.g. lacZ) in these transgenic mice. An alternative approach for region specific htTA expression was to use the CaMKIIα promoter but to repress the forebrain-specific promoter in regions other than hippocampus by the neuron-restrictive silencer element (NRSE) of the N-methyl-D-aspatate receptor 2C (NR2C) subunit. The expression pattern and activity of the htTA under control of the NR2C silencer-containing chimeric promoter were observed restrictively in hippocampus of two lines (sCN1 and CN10), namely only in DG, and in CA1 and DG, respectively. Activity of the htTA in the CN10 line was detected at postnatal stage and was turned off completely by doxycycline.

It is demonstrated here that the humanized tTA (htTA) provides efficient expression and reversible inducibility *in vitro* and *in vivo* and that the NR2C silencer-containing chimeric promoter was successfully applied in transgenic mice. The hippocampus-specific genetic switch by the htTA should make it possible to study genes of interest involved in hippocampal functions and should advance the research of gene function in the CNS (central nervous system).

1. Introduction

It is now possible to introduce a defined, cloned gene into germline of an animal and to analyze its role and regulation within the whole transgenic organism. The transgenic mouse is the model system of choice in many physiological and biological studies. In particular, transgenic mice have been chosen as models for neuronal gene analysis and neurodegenerative diseases. Contemporary analysis of gene function in the whole organism rests predominantly on the technology of genomic manipulations of mice. Cloned gene delivery to mouse oocytes or embryonic stem cells permits stable alteration of the mouse genome. However, strategies of inducible somatic mutagenesis are required so that gene function can be studied in a chosen cell type at a chosen time. Our goal of this study is to optimize an inducible system for its use in mice and to generate transgenic mice expressing the improved inducible system in a more spatial manner in the central nervous system (CNS) for the investigation of neurobiological questions.

1.1. Inducible gene expression systems

Gene function during development has been often studied by making stable, uninducible changes to the genome. This approach has a major drawback in that a mutant organism may compensate for the loss of a gene product without an apparently altered phenotype or may yield a complex, uninterpretable phenotype. Moreover, if the genetic modification or complete loss of a gene by conventional genetic manipulation engenders embryonic or neonatal lethality, gene function at late stage of development cannot be analyzed. It would be highly valuable if the expression of a particular gene could be both temporally and spatially restricted through the use of an inducible genetic system. Various inducible genetic expression systems, by which gene expression could be turned on or off at will, have been developed. Irreversibly and reversibly inducible gene expression systems derived from simple eukaryotes and prokaryotes are described below.

1.1.1. Irreversible system by Cre or FLP recombinase

Somatic mutagenesis resulting from precise genetic modification can be efficiently achieved by using P1 phage Cre recombinase to catalyze excision or inversion of DNA flanked with specific 34 bp sequences termed loxP site (Hoess et al. 1986 and Sauer 1998). An alternative system for site-specific recombination is yeast Flp recombinase and FRT (Flp recombinase target) sites; however its efficiency is relatively low in mammalian cells because of different physiological temperature of yeast and mammals (Senecoff et al. 1988) (Figure 1A and B).

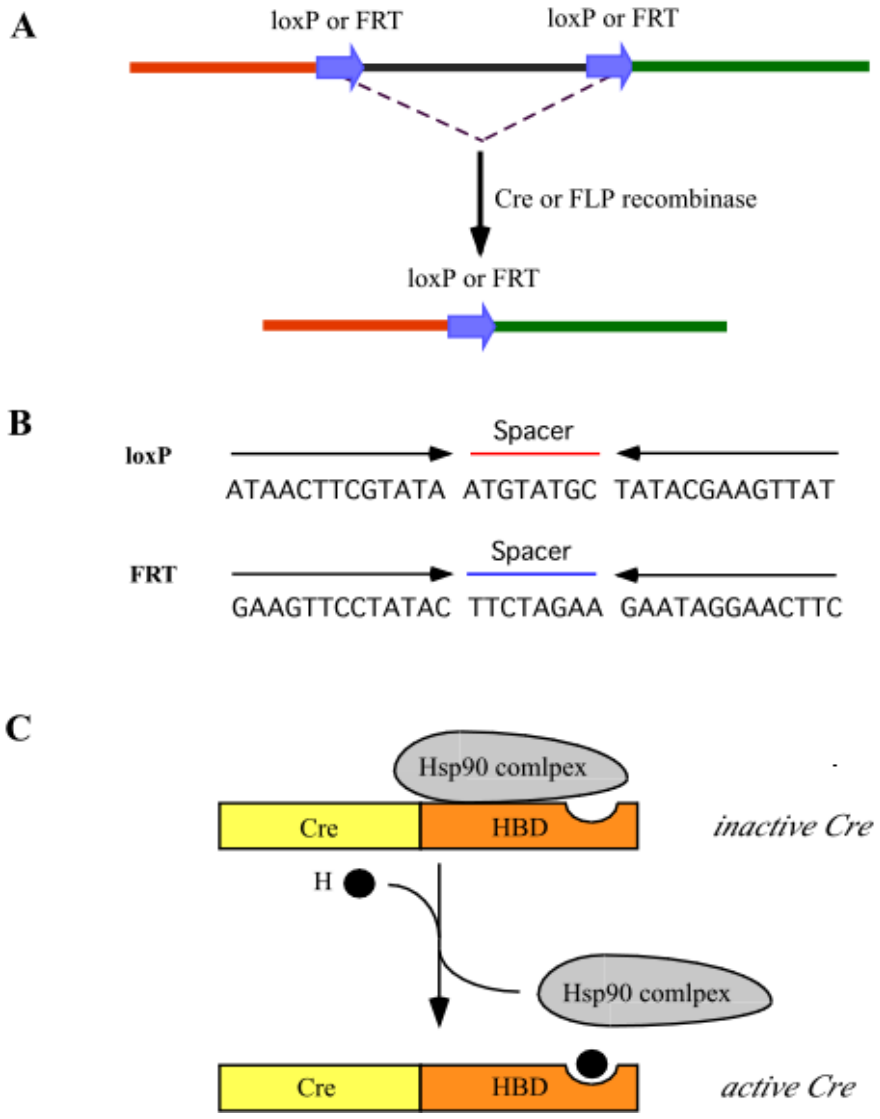

Figure 1. Cre or FLP-mediated recombination and schematic representation of the regulation of Cre recombinase activity. (A) The genomic region flanked with two directly repeated loxP or FRT can be excised by the Cre or FLP recombinase. (B) lox P and FRT sites are composed of the spacer and inverted repeat indicated by thin arrows. (C) An activity of Cre recombinase can be regulated by fusion to the hormone-binding domain (HBD) of a steroid receptor. The fused Cre recombinase along with the hormone-reversible heat-shock protein 90 (Hsp90) complex is poattranslationally inactive. By hormone binding Hsp90 complex can be released from the Cre fusion protein and the Cre recombinase is in active state.

The genetic switch mediated by the Cre-loxP system is irreversible. Therefore temporal regulation of Cre recombinase expression has been attempted during past years. For this, Cre recombinase was fused with mutated steroid hormone-binding

domain (HBD) of progesterone or estrogen receptor, which is responsive not to endogenous hormones, under a control of tissue-specific promoter (Pcard 2000) (Figure 1C). The fusion protein becomes active by the synthetic ligands RU486 or tamoxifen, however, both RU486 and tamoxifen are prone to potential side effects resulting from interference with the endogenous hormone circuit such as processes of mammary gland or reproductive development (Kellendonk et al. 1996 and 1999, Brocard et al. 1997 and 1998, Danielian et al.1998). Second, local administration of adenovirus expressing Cre recombinase demonstrated toxicity and immunogenicity mediated by adenoviral infection (Akagi et al. 1997 and Anerson et al. 1998). The combination of Cre-loxP system and tetracycline-controlled regulatory system, which will be subsequently described, could be promising for Cre-based gene regulatory system in a conditional manner (Utomo et al. 1999).

1.1.2. Reversibly inducible systems derived from eukaryotic regulatory system

The inducible systems, based on endogenous system from simpler eukaryotic organisms (e.g. *Drosophila melanogaster*, *Caenorhabditis elegans*), usually suffer from high basal level of activity in non-induced state and divers side effects, because the inducer activates not only a responsive transgene under a study but also undesired endogenes. Additionally there is a lack of a spatial control of a transgene and thus a non-specifically regulated transgene is expressed in all cell types. Such drawbacks of theses systems limited utility in eukaryotic organism for example in mice.

(1) Heat shock

Some heat shock protein promoters (*D. melanogaster hsp*70, *C. elegans hsp*68) which rely on endogenous heat shock transcription factors for their activity were used in cell lines (Schweinfest et al. 1988) and transgenic mice (Kothary et al.1989). The activity of these promoters has no basal level of gene expression at 37^{o}C and induced with fast kinetics (within minutes) by a temperature shift from 37^{o}C to 42^{o}C. In spite of a stringent regulation, the induction system has drawbacks such as a poor induction ratio (~10 fold) with many pleiotropic effects through heat stress and no spatial regulation of a transgene.

(2) Heavy metal ion

The inducible system based on promoters responsive to heavy metal ions, particularly Cd^{2+} and Zn^{2+} showed a significant leakiness, the modest levels of induction, no cell type-specific control, and the toxicity associated with administration of heavy metal (Filmus et al. 1992).

(3) Interferon

Gene expression under a control of interferon-inducible regulatory element e.g. a promoter of Mx1 was activated rapidly in mice by injection, double-stranded RNA, or virus (Arnheiter et al. 1990, Kühn et al. 1995). However, the level of induction and background was variable in different tissues as well as in from mouse to mouse, because of different interferon availability in various tissues and variable production of endogenous interferon during viral infections and other illness. Additionally, an induction leads to biological side effects by treatment with interferon α/β or dsRNA.

(4) Hormones

The system by using steroid hormone (e.g. glucocorticoid, estrogen, and progesterone) inducible promoter exhibits a high leakiness of the inactive state and pleiotropic effects as above systems (Friedman et al. 1989, Braselmann et al. 1993, and Wang et al. 1994). Because a number of mammal endogenous promoters have a hormone-responsive element, undesired endogenes as well as a transgene could be expressed by hormone induction. It might make difficult to figure out definite conclusion of effects of a transgene under a study.

(5) Ecdysone

The insect steroid hormone ecdysone-inducible gene expression system has been described as an alternative and promising system for use in mammalian cells and transgenic mice (Yao et al. 1993). The ecdysone triggers metamorphosis in *D. melanogaster* mediated via a heterodimer of the ecdysone receptor (EcR) and ultraspiracle (USP). The heterodimer transactivates the ecdysone response elements (EcREs)-containing promoter composing of two inverted half-sites of the sequence AGGTCA spaced by 1 nucleotide. To improve sensitivity of an ecdysone-inducible system in mammalian cells, the USP was substituted by the retinoid X receptor (RxR), the mammalian homologue, and the EcR was modified. The N-terminally truncated EcR was fused with the herpes simplex virus protein VP16 activating domain (VpEcR). The DNA-binding specificity of VpEcR was also altered to mimic that of the glucocorticoid receptor (GR), which binds to an inverted repeat of the sequence AGAACA spaced by 3 nucleotides. The DNA-binding specificity was improved by mutating 3 amino acids residues of the DNA binding domain of VpEcR and by modifying the tandemly arranged ecdysone response elements (EcREs) containing two different half-sites, AGGTCA and AGAACA spaced by 1 nucleotide. This modified receptor is called the VgEcR and combined with the RxR (Figure 2). Thereby induction levels were increased to four orders of magnitude by treatment

with ecdysone, the synthetic analogue muristerone A (murA) or ponasterone A, which is found by screening natural plant compounds for improvement of inducibility, while maintaining a very low basal activity (No et al. 1996, Albanese et al. 2000, Yu et al. 2000). In this system, tissue-specificity can be conferred by choice of promoters that direct expression of the ecdysone receptors. The ecdysone-inducible system had no apparent side effect through an administration of ecdysone or murA, high inducibility with a very low leakiness. In addition, the lipophilic nature of ecdysteroid allows penetrating into all tissues, including the brain with negligible storage and expeditious clearance. Nevertheless the ecdysone-inducible system has a limitation of the requirement for three transgenes.

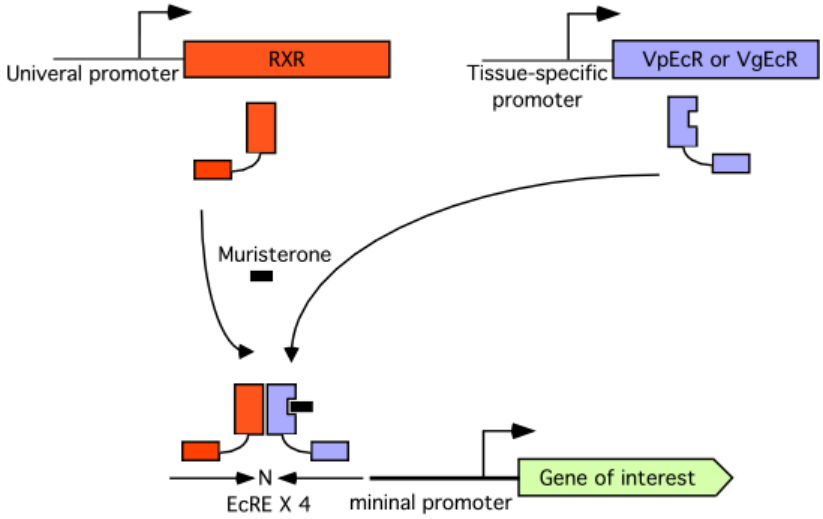

Figure 2. Schematic diagram of the ecdysone-inducible gene expression system. The retinoid X receptor (RxR), the mammalian homologue of ultraspiracle (USP), and the modified ecdysone receptor VpEcR (or VgEcR) can be expressed under a control of tissue-specific promoter and heterodimerize. The heterodimer can transactivate the ecdysone response elements (EcREs)-containing promoter in the presence of hormone or synthetic analogue muristerone. The EcREs are placed upstream of a minimal promoter, which can drive the expression of gene of interest. Then an ecdysone-responsive promoter can control expression of gene of interest. The EcRE is composed of tandemly arranged inverted sequences spaced by 1 nucleotide (indicated by arrows and N for spaced nucleotide).

1.1.3. Inducible systems based on prokaryotic regulatory elements

Highly specific control of gene activity in higher eukaryotic cells has been achieved by the utilization of the well-characterized prokaryotic regulatory elements because regulatory elements and inducer do not rely on endogenous control elements. The inducible systems based on two well-studied regulatory elements from *Escherichia coli*, lac and tet operones, have been shown as monospecific regulatory circuits in mammalian cells and transgenic mice. The use of tissue-specific promoter for expression of these regulatory elements can contribute ability of a spatial regulation in transgenic mice.

(1) lac Repressor/Operator-based inducible system

The *E. coli* lac operon-based system composes of two components; a lac repressor (lacR) and a DNA sequence called lac operator (lacO). In this system, the lac operator and repressor provide tight, reversible transcriptional control of genes involved in the uptake and metabolism of lactose (Figure 3A). The lac repressor binds to operator (lacO) and prevents formation of an initiation complex RNA polymerase and the promoter. This repression can be specifically reversed by the synthetic and nonmetabolizable inducer isopropyl β-D-thiogalactopyranoside (IPTG). The most straightforward approach followed the prokaryotic paradigm: lac operator sequences were placed near the TATA-box or start site of mammalian promoter. Thereby, a lacR-O complex within the promoter region would directly interfere with initiation of transcription of an endogene. The transcription from promoters containing lacO sequences could indeed be regulated in mammalian cells by IPTG (Hu and Davidson 1987 and Brown et al. 1987). During past years, few studies were established by the lac-operon based inducible system, no in transgenic mice, because of some limitations e.g. cytotoxic levels of IPTG required for induction, low intracellular concentration of the repressor protein to achieve a efficient induction in the mouse. Currently, Caronin and colleagues (2001) described the successful application of a functional *lac* operon-regulatory system in the mouse (Figure 3). In their study, the lac repressor was optimized concerning splicing, translational codon, and methylation-associated silence. Three lac operators were inserted into the tyrosinease promoter so that it is directly regulated by the ubiquitously expressed lac repressor. The lac repressor could repress the activity of a reporter gene, the mouse tyrosinease coding sequence, which subsequently could be derepressed by IPTG at a noncytotoxic level. The successful transfer of a lac operator-repressor gene regulation system to the mouse promises reversible control of the endogenous genome.

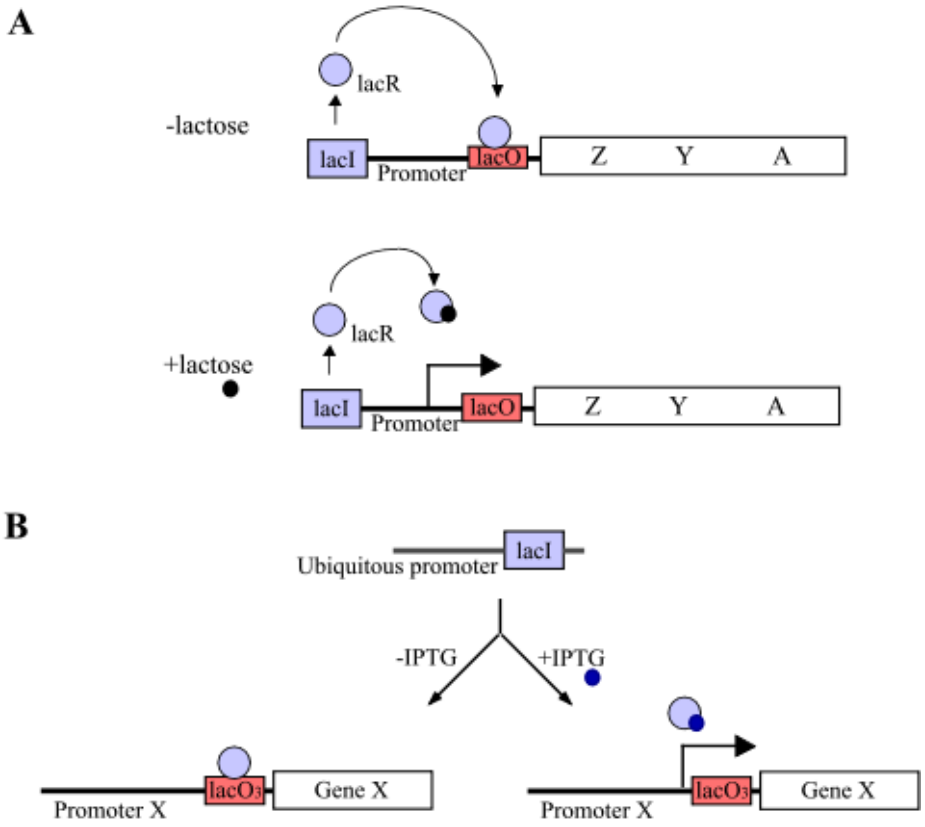

Figure 3. The lac operon-based inducible system. (A) The lac operon in *E. coli* operates by a repression mechanism. In the absence of lactose (upper panel), the lac repressor (lacR) binds to lac operator (lacO) and prevents gene expression. In the presence of lactose (down panel), the lacR undergoes a conformational change and results in the derepression of genes. (B) Schematic representation of lac operon-based inducible system in transgenic mice. The lac repressor is ubiquitously expressed. The lac repressor represses the expression of gene X by binding to the lac operator sequences, which are were placed near the start site of promoter of gene X. This repression can be specially by the synthetic inducer isopropyl β-D-thiogalactopyranoside (IPTG); lacI, gene encoding lac repressor; lacO, operator sequence; Z, Y, A, genes encoding β-galactosidase, permease, and transacetylase, respectively, needed for uptake and utilization of lactose.

(2) Tetracycline-controlled transactivator (tTA) system

The tetracycline-controlled transactivator (tTA) is the fusion protein between the repressor (tetR) of the Tn10 tetracycline resistance operon of *E. coli* and the C-

terminal transcriptional activating domain of VP16, known to be essential for the transcription of the immediate early viral gene (Gossen et al. 1992) (Figure 4A). This transactivator binds to operator sequence (tetO) of the tet operon in a tetracycline-dependent manner: When tTA-responsive promoter (P_{tet}), fused between seven tetO sequences and a minimal RNA polymerase II promoter sequences derived from the human cytomegalovirus immediate-early (IE) promoter (P_{hCMV}), is placed in front of a gene, the tTA mediates on / off situation of gene transcription in presence or absence of tetracycline or its analogue (Baron U. et al. 2000, Mansuy et al. 2000) (Figure 4B). The bi-directional promoter, in which the seven tetO sequences ($tetO_7$) are flanked with two minimal promoters, allows co-regulation of two genes (Baron et al.1995, Krestel et al. 2001).

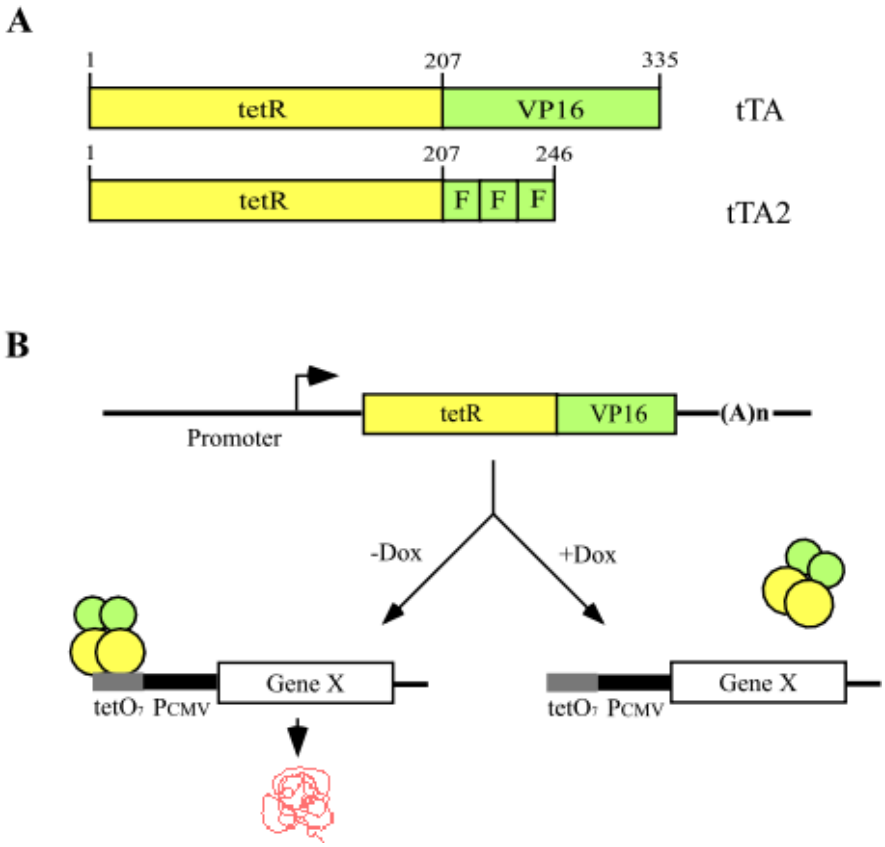

Figure 4. The tetracycline-controlled transactivators (tTA) and schematic outline of tTA regulatory system. (A) The tTA is fusion protein between tet Repressor (tetR), consisting of 207 amino acids, and the 128 – amino acids transactivation domain of VP16. In tTA2, VP16 domain is changed with three minimal activation domains (F domain). (B) Mechanism of the tTA regulatory system. The tTA binds in absence of the effector molecule doxycycline (Dox) to an array of seven tet operator sequences (tetO7) placed upstream of a minimal promoter and

activates transcription from a minimal cytomegalovirus promoter (PCMV), which itself is inactive. But not in presence of Dox.

The tTA system is one of the most efficient inducible gene regulatory systems for use in mammalian cells, *D. melanogaster* (Girard et al. 1998), plant (Weinmann et al. 1994, Zeidler et al. 1996) and transgenic mice (Kistner et al. 1996, Ewald et al.1996, Mayford et al.1996). The distinct advantages of tTA system are (1) the high induction ratio (~10^5), combined with very low basal leakiness, (2) the specificity of the tetR for its operator sequence, the high affinity of tetracycline for the tetR, (3) well-characterized pharmacological properties of tetracycline and its analogues, which is able to penetrate most cell boundaries (Hillen and Berns 1994), (4) low toxicity of tetracycline and its derivatives, and (5) a genetic switch that would permit the control of individual gene activities reversibly in a temporal manner.

With these advantages, the tTA system has been extensively applied in different tissues, organs of transgenic mice. In neuroscience, the conditional gene expression system by using tTA has been employed to outline the modelling, therapeutic strategies of neuropathology and to study the molecular mechanisms underlying the processes of learning and memory. For instance in prion disease, the cellular prion protein (PrP^c) is converted into the pathogenic isoform (PrP^{sc}) via a posttranslational modification (Prusiner 1997). The expression of PrP^c in transgenic mice was regulated by tTA under a control of the PrP gene promoter (Tremblay et al. 1998). It was shown that repression of PrP^c expression on doxycycline in young adult transgenic mice (tTA:PrP) is not deleterious, whereas accumulation of PrP^{sc} in same line of animal is lethal. Mice expressing a mutated huntingtin fragment controlled by tTA also lead to neuropathologic signs characteristic of Huntington's disease and these symptoms could be reversed by suppression of the transgenic expression with doxycycline (Yamamoto et al. 2000). In the study of synaptic plasticity concerning learning and memory, transgenic mice carrying tTA under a control of the α-type calcium-calmodulin-dependent kinase II (CaMKIIα) gene promoter has been constructed and the tTA directed the expression of mutated Ca^{2+}-inddependent form of CaMKIIα in transgenic mice (Mayford et al. 1996). Inducible expression of mutant CaMKIIα resulted in a loss of hippocampal LTP and a deficit in a spatial memory. Suppression of transgene expression by an administration of doxycycline reversed both the physiological and the behavioural phenotype. Currently, Krestel et al. (2001) showed that the tTA-sensitive bidirectional expression module is well suited to express genes of interest in a co-regulated manner in mouse. In the study of Mack et al. (2001), expression of green fluorescent protein (GFP)-tagged GluR A could be controlled in GluR A-deficient mice by using CaMKII-tTA mice (Mayford et al. 1996). It was reported that hippocampal LTP was rescued in CA1 pyramidal cells of adult GluR A-deficient mice by constitutive or induced expression of tTA-controlled

^{GFP}GluR A. Theses studies strongly indicated that the tTA-mediated gene regulatory system has become a powerful tool for utility in mice.

1.2. Why 'humanised tTA (htTA)' is needed

Despite of a number of trials to create tTA mice, so far the useful and functional tTA mice in brain are the CaMKII-tTA (Mayford et al. 1996), enolase-tTA (Chen et al. 1998) and prion-tTA mice (Tremblay et al. 1998). It is difficult to generate transgenic mice expressing the tTA to direct efficiently a gene of interest in a region specific manner in brain. Unfortunately the trials of construction of tTA mice failed to present us useful tTA mice even by knock-in approach using GluR B, NMDA receptor 1 and ADARA2 (Jerecic et al. 1999). The tTA gene appear to be incorrectly spliced or expressed too deficient to induce tTA responsive gene (e.g. lacZ) in these transgenic mice. Therefore we attempt to increase the translation efficiency and mRNA stability of tTA derived from prokaryotic element in eukaryotic environment, termed 'humanised' tTA (htTA). The humanised Green Fluorescent Protein (GFP) from jellyfish (Zolotukhin et al. 1996), Cre recombinase and the currently reported *lac* repressor (Cronin et al. 2001) have been already shown stability and high level expression of these adapted protein in mammalian cell. In order to increase the tTA expression in eukaryotic environment, 'humanized tTA (htTA)' was designed on the DNA sequence level without change of amino acid sequence regarding eukaryotic codon usage, elimination of potential splicing signal, avoidance of CG sequence, and introduction of kozak sequence. In addition the tTA 2 gene, whose VP16 moiety is replaced by three minimal critical activating domains (F domain) was used as a template of a design of htTA (Baron et al. 1997, Regier et al. 1993). The modification of the activating domain of tTA 2 allows tolerance at higher intracellular concentrations, whereas overexpression of transcription in general results in squelching (Gill and Ptashne 1988).

1.3. Generation of transgenic mice expressing htTA in a subregion-specific manner in brain compared to previous CaMKII-tTA mice

Next, we tried to achieve transgenic mice expressing the optimized htTA in a more spatially specific manner in brain for investigation of brain function at molecular and cellular level. The brain subregion-restricted gene regulation should allow a more precise analysis of gene function under a study.

Previously, the CaMKIIα promoter has been the most frequently used the promoter for control of expression of several inducible elements in transgenic mice showing its activity in relatively widespread forebrain of transgenic mice. The forebrain-specific

CaMKIIα promoter has been used to direct tissue-specific expression of the tTA (Mayford et al. 1996), rtTA (Malleret et al. 2001, Mansuy et al. 1998) and Cre recombinase (Minichiello et al.1999, Tsien et al. 1996) in rodent brain. The expression pattern of tTA under a control of CaMKIIα promoter has been determined in the neurons of the neocortex, the hippocampus, the striatum and septum, the amydala, and the basal ganglia by in situ hybridization (Mayford et al. 1996) (Figure 5). Additionally, tTA activity was found in thalamus, pons, medulla oblongata, spinal cord, and eyes with weaker signals by generating new sensitive tTA-reporter GFP lines (Krestel et al. 2001). In the αCaMKII-rtTA transgenic mice, it expression was found in CA1 and CA2 area of the hippocampus with almost no signal in CA3 region, in dentate gyrus, in superficial layers and deeper layer of cortex and in the striatum and septum by lacZ staining (Mansuy et al, 1998). The Cre recombinaes under a control of CaMKIIα promoter was observed in the hippocampus with strong signal in CA1, lighter signal in CA3 and dentate gyrus, the neocortex, the striatum, and amydala (Minichiello et al, 1999). Tonegawa group generated αCaMKII-Cre transgenic mice, however, the Cre expression was relatively late in development and mainly restricted in the hippocampus due to different condition of transgene integration (Tsien et al. 1996).

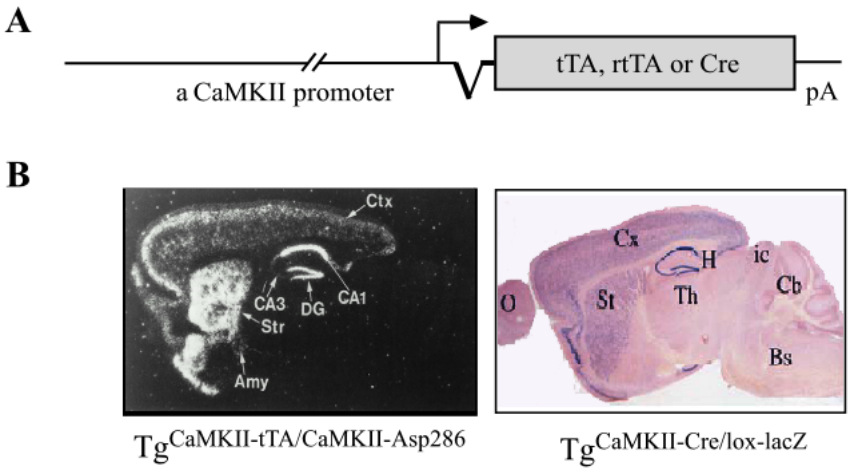

Figure 5. The expression of transgenes under a control of CaMKIIα promoter. (A) Schematic draws of the CaMKIIα promoter-controlled transgenes. The arrow indicates the transcriptional start of CaMKIIα promoter. Triangle represents synthetic intron sequences. pA, polyadenylation signal. (B) Examples of CaMKIIα promoter-controlled expression patterns in brain. *Left*; In situ hybridization of CaMKII-Asp286 mRNA directed by CaMKII-tTA. *Right*; Spatial pattern of Cre activity in double transgenic mice of CaMKII-Cre:lox-lacZ by X-gal staining.(published by Mayford M. et al.1996 and Minichiello L. et al. 1999);Cx, cortex; CA1/3, areas of Ammon's horn; DG, dentate gyrus; Amy, amygdala; H, hippocampus; Cb, cerebellum; Str, striatum; Th, thalamus; ic, inferio colliculus; O, olfactory bulb; Bs, brain stem.

Together, the CaMKIIα promoter has been useful to express transgenes in forebrain, but ultimately a transgenic mouse containing different tissue-specific or more specific expression patterns of the inducible regulatory elements could be needed depending on numerous genes of interest. The hippocampus has been one of the most frequently employed areas of the central nervous system as a model system for the study of neurobiological questions (Figure 6). There are two main reasons. First, its distinctive and identifiable structure. The apparent simplicity of the hippocampal neuronal circuitry is attractive at anatomical, physiological, and molecular biological level. Second, investigations of a role of hippocampus. The hippocampus may affect some neurodegenerative diseases (e.g. Alzheimer's disease), certain pathological conditions (e.g. epilepsy) and play a fundamental role in some forms of learning and memory.

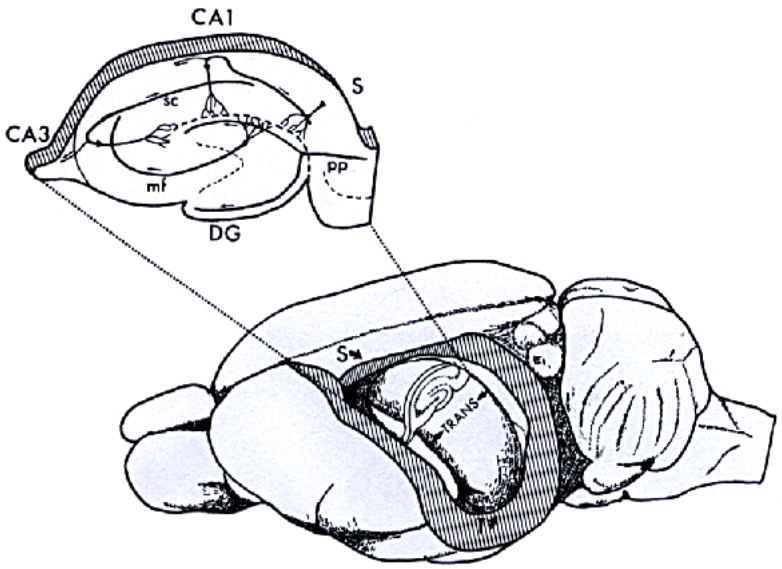

Figure 6. Simplified drawing of the location and the intrinsic connection of hippocampus of rat brain. The hippocampus is a banana-shaped structure that extends from the septal nuclei rostrally to temporal cortex, caudally. The long axis is called the *septotemporal axis* (indicated by S-T) and the orthogonal axis is the *transverse axis* (TRANS). A slice cut perpendicular to the long axis of the hippocampus (above left) shows several fields of the hippocampal formation and several of the intrinsic connections. Abbreviation: DG, dentate gyrus; CA1, CA3, areas of Ammon's horn (cornu Ammonis); S, subiculum; pp, perforant path fibers from enthorhinal cortex; mf, mossy fibers from the granule cells; sc, Schaffer collateral connections from CA3 to CA1. (published by Amaral D.G: and Witter M.P. 1989)

For the study of hippocampal functions, affects from the other region of brain should be eliminated by expressing the inducible element in a hippocampus-specific manner.

For that we had two approaches by using KA1 bacterial artificial chromosome (BAC) and applying neuron-restrictive silencing element (NRSE). By using KA1 bacterial artificial chromosomes (BAC), it was expected that expression of the htTA under the control of KA1 promoter is directed in the areas expressing KA1 itself, namely in CA3 and dentate gyrus (DG) of hippocampus. An alternative approach was to repress extra hippocampal expression of the CaMKIIα promoter by the neuron-restrictive silencer element (NRSE) of the N-methyl-D-aspatate receptor 2C (NR2C) subunit.

2. Results

2.1. Design and construction of the humanized tTA (htTA)

To increase the expression level of tTA in eukaryotic cells, the humanized tTA was designed on the DNA level in terms of (1) substitution of codons by their preferentially used degenerate codons in eukaryotic cell, (2) removal of potential splicing signals, (3) avoidance of CG dinucleotide sequences, which tend to be methylated and possibly prevent active gene expression, and (4) introduction of a Kozak consensus sequence, which could enhance translation efficiency of the mRNA (Kozak M. 1984), next to the start codon AUG. The tTA2, which has 100 % relative activation potential with a lower cytotoxicity, was used as template because it is tolerated at 3-fold higher concentration in cells. This is due to its VP16 activation domain being reduced to three acidic minimal activation domains (F domains), each consisting of only 13 amino acids (Baron et al. 1997) (Figure 7). To maximize the functional efficacy of the transactivator on the responsive gene in the nuclear compartment, the nuclear localization signal (NLS) was introduced between tetR and three F domains. The htTA was synthesized by assembly PCR with overlapping oligos according to the method of Stemmer et al. (1995) (Figure 8). The synthesized htTA fragment (784 bp) was cloned into pBluscriptIISK(+) and verified by sequence analysis.

2.2. Activation potential and comparison of the htTA in transiently transfected cells

To asses the activation efficiency of the htTA without and with the nuclear localization signal (NLS) compared with the prokaryotic tTA, the tTA, htTA-NLS and htTA+NLS genes were subcloned into the expression vector pRK5 (Schall et al. 1990). HeLa X1/6 cells containing a chromosomally integrated tTA-responsive luciferase gene as a reporter (Gossen et al. 1992), were transiently co-transfected with plasmids encoding the tTA, htTA-NLS and htTA, respectively and β-galactosidase (pCMVpnlacF, Mercer et al. 1991) for a normalization. The transfected HeLa X1/6 cells were incubated in the absence or presence of doxycycline (1μg/ml) for 30 hrs. Luciferase activity was detectable by use of the luminescent assay (luciferase gene assay kit, Dual-Light, TROPIX) and normalized to β-galactosidase activity (Table 1). An increase of induction efficiency of the humanized tTA measurable by luciferase activity was observed and this activity was effectively abolished by doxycycline: the htTA-NLS reaches 133% and the htTA+NLS 298% of the activity conferred by prokaryotic original tTA in HeLa cells. The htTA+NLS generates almost 3-fold higher

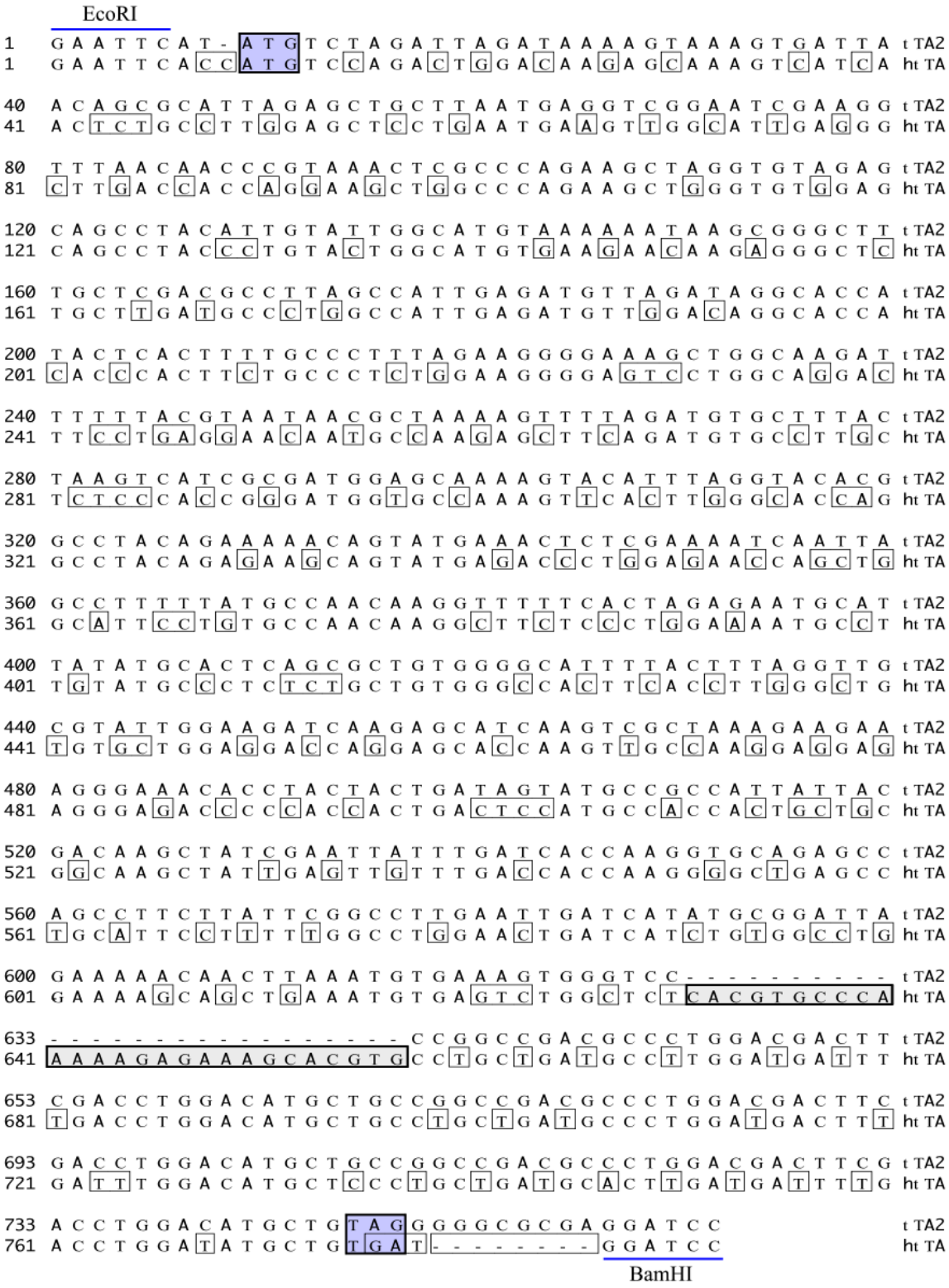

Figure 7. Alignment of DNA sequences of the tTA2 and humanized tTA gene. Nucleotides differing from the prokaryotic tTA2 were marked with box on the htTA sequences. Two blue boxes indicate translational start and stop codon, and the nuclear localization signal (NLS) is in a grey box. Underlines show restriction sites, which were used for subcloning.

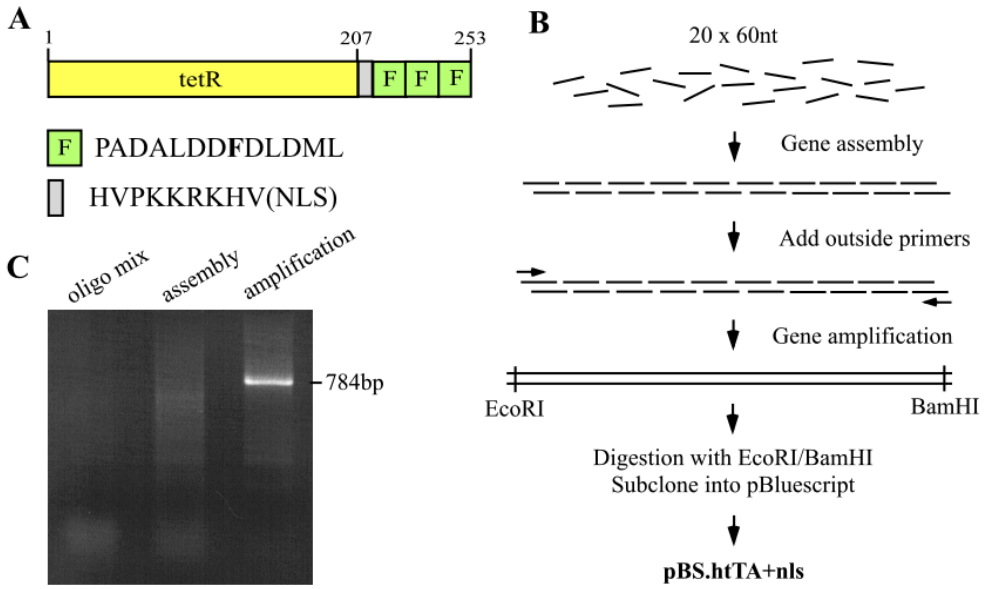

Figure 8. Design and synthesis of the humanized tTA. (A) Representation of the htTA fusion protein containing three F domains and nuclear localization signal (NLS) between humanized tetR and three F domains. The number indicates position of amino acids and capital letters are codons with single letter amino acids. (B) Schematic procedure for the htTA gene synthesis by oligo shuffling. Twenty overlapping oligos (approximately 60 nucleotides in length) on DNA sequences of the htTA gene were assembled by PCR and subsequently amplified with two outside primers (arrows). The outside primers contained EcoRI endonulclease site for 5' end and BamHI for 3' end. The amplified fragment was subcloned into pBluscriptIISK(+) via EcoRI and BamHI digestion. (C) 1 % agarose gel shows oligo mixture before the PCR assembly (left), smear assembly PCR products in variable length (middle) and the 748 bp fragment amplified with the outside primers.

induction while maintaining a very low basal activity and tolerance at higher intracellular concentration. Thereby the htTA+NLS could be confirmed as one of the best tetracycline-controlled transactivators for an application in transgenic mice.

Table 1. Comparison of induction efficiency of transcriptional activation between the synthesized htTAs and prokaryotic tTA

	Luciferase activity (RLU/μg protein)		Relative Activity (%)	Plasmid
	+dox	-dox		
tTA	0.1	1580	100	PRK5.tTA
htTA-NLS	0.26	2107	133	PRK5.htTA-nls
htTA+NLS	0.12	4716	298	PRK5.htTA+nls

Rates were obtained light emission/μg protein. The luciferase activities were measured by use of the luminescent assay in three independently transfected HeLa cells, HeLa X1/6, which contain the luciferase gene under the transcriptional control of the chromosomally integrated tTA-dependent promoter P_{hCMV-1} (Gossen and Bujard 1992). HeLa X1/6 cells were transiently transfected with plasmids encoding the tTA, htTA-NLS, and htTA+NLS, respectively and cultured in the absence or presence of doxycycline (1 μg/ml) for 30 hrs. These values were normalized by co-transfection of plasmid encoding β-galactosidase (pCMVpnlacF, Mercer et al. 1991), which were assayed either simultaneously by luminescent assay or by standard liquid O-nitrophenyl β-galactoside assay (ONPG, Sigma), and related to the activity of previously described tTA (100 %); RLU, relative light units; dox, doxycycline.

2.3. Generation of transgenic mice expressing the htTA in a region (hippocampus)-specific manner in brain

In our attempt to develop a subregion-restricted regulatory system in the mouse brain for study of molecular mechanisms of brain function, the improved htTA was used. To achieve more region-restricted expression patterns of the tTA inducible system, especially in hippocampus, compared with the forebrain-specific CaMKII-tTA mouse (Mayford et al. 1996), we had two approaches: (1) using bacterial artificial chromosome (BAC) harbouring the 5' regulatory region of the high-affinity kainate receptor subunit KA1 gene, and (2) applying the silencer region of N-methyl-D-aspatate receptor subunit 2C (NR2C).

2.3.1. Generation of KA1.htTA transgenic mice

To generate transgenic mice expressing the tTA-inducible system in a region-specific manner similar to the expression pattern of the high-affinity kainate receptor subunit KA1, the htTA+NLS was inserted into the ATG-including exon of the KA1 gene through homologous recombination of a KA1 BAC clone. In addition, the tTA was directed via the same way for comparison of mRNA stability and activities *in vivo* between the htTA and the tTA.

The KA1 gene has a highly restricted expression mainly in pyramidal cells of the hippocampal CA3 and granule cells of the dentate gyrus of adult rat brain determined through *in situ* hybridiztion (Werner et al. 1991). Kask et al. (2000) generated transgenic mice expressing Cre recombinase under a control of the KA1 promoter by using 550 kb YAC (yeast artificial chromosomes). It was shown that expression pattern of Cre recombinase controlled by the KA1 promoter carried on 550 kb YAC resembles that of endogenous KA1. The 5´ regulatory elements of the KA1 gene could accommodate to direct the htTA gene in a region-specific manner as the KA1 gene itself in transgenic mice. Now we used bacterial artificial chromosomes (BACs) carrying the mouse KA1 gene because there are several advantages of using BACs, compared to YACs. (1) BACs have a high stability in terms of their propagation in recombination deficient *E. coli* host cells, (2) isolation, purification and handling of BACs are less difficult since they exist as supercoiled circular plasmids, (3) a direct sequencing can be applied to BACs, (4) BACs can be modified in *E. coli* to insert transgenes at a desired position. and (5) a large size of the insert in BACs approximately 150 kb ~ 200 kb might provide for less integration dependency of transgene expression. Recently, transgenes were obtained successfully by modifying

BACs (Antoch et al. 1997, Jessen et al. 1998, Nielsen et al. 1997, and Probst et al.1998).

2.3.1.1. Genomic structure of the mouse KA1 gene

The cDNA from rat brain and human encoding the KA1 subunit have been cloned (Werner et al. 1991, Herb et al. 1992 and Kamboj et al. 1994) but its detailed genomic structure is not delineated. The Celera data bank search provided genomic sequence information of the mouse and human KA1 gene. The comparison of amino acid sequences at the KA1 cDNA level of mouse and human shows high percent of identity among species; 99.5 % of similarity of amino acid sequences between rat and mouse, and 97.5 % between mouse and human. The Celera data bank mouse genome accession x5J8B7W3WPH and human genome accession x54KRE8WCJ9 revealed that the gene of KA1 encoding 956 amino acids exists as a single copy on mouse chromosome 9 and on human chromosome 11, respectively and is divided into 19 translated exons both in mouse and human (Figure 9A). The mouse KA1 gene spans about 300 kb between the first ATG exon (132 bp) and the last exon 19 and within are some very large introns contained: one of 124 kb in a size between the first ATG exon and exon 2, three of approximately 25 kb between exon 6-7, exon 9-10, and exon 11-12. The human genome Celera data revealed that the organization of exons and introns of the KA1 gene is highly conserved between human and mouse. The locations of exon-intron boundaries are at the exactly same positions on the mouse and human KA1 open reading frame (OFR) and the length of introns is very little variable (Figure 9B). It is reported that there is one untranslated exon (27 bp) upstream of the ATG exon in the rat cDNA sequence. The 27 bp untranslated exon (exon0) was identified 8.2 kb upstream of the ATG exon1 by southern blot analysis of mouse genome BACs and by Celera mouse genomic data with 2 different nucleotides compared to the rat cDNA sequence (Figure 9C and D). In human genome, no untranslated exon was yet identified.

2.3.1.2. Screening of BACs library for mouse KA1 gene

To isolate BACs containing the mouse KA1 gene, in particular 5' regulatory region and the ATG exon, the 682 bp of PCR amplified fragment covering the partial introns surrounding the ATG exon was used for screening of the BAC library of the mouse C57BL/6 strain (Genome Systems, St. Louis, MO, USA). Two KA1 BAC clones were isolated and pulsed-field gel electrophoresis (PFEG) of NotI-digested BAC DNA revealed that two BAC clones contain inserts of approximately 166 kb and 60 kb in a size, respectively. Direct BAC sequencing and the Celera data bank search revealed that the location of the 166 kb KA1 BAC is on the 5' regulatory region of the

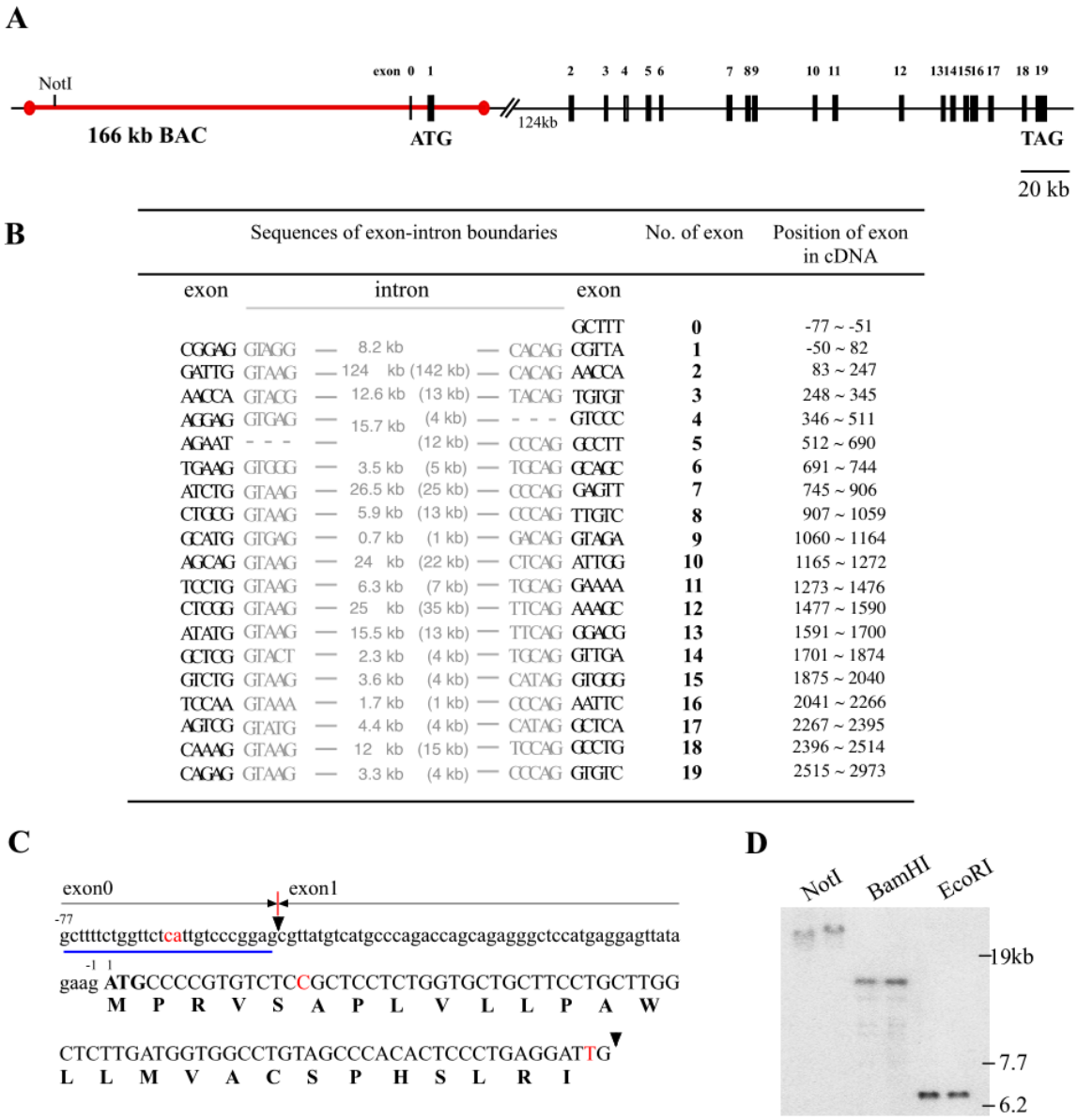

Figure 9. The exon-intron organization of the mouse KA1 gene. (A) Schematic representation of the mouse KA1 genomic structure according to the Celera data bank search. Exons of the KA1 gene are shown by filled boxes and numbered (exon0-19). Open box indicates the exon4, which was not found in mouse genomic sequences because of short segments unidentified by the Celera. Translational start codon (ATG) on the exon1 and stop codon (TAG) on exon 19 are indicated. Red line shows the location of 166 kb BAC harbouring the mouse KA1 gene. There is one NotI site within the 500 kb gene. (B) the nucleotide sequences of the exon-intron boundaries of the mouse KA1 gene. Intron sequences and length of the mouse KA1 gene are typed in grey. Additionally the length of introns of the human KA1 gene is in brackets. Position of exons is numbered on the mouse KA1 cDNA starting from translational start site. (C) The DNA sequences of exon0 and exon1 on the mouse KA1 cDNA and amino acid sequences (capital single letter under DNA sequences) of exon1. Black arrowheads indicate the boundary between exon0 and exon1; Red letters, different nucleotide

from rat; number, position of nucleotides. Blue underline shows the exon0 and was used as a probe for southern blot analysis of the BAC containing the 166kb mouse KA1 gene (D). NotI, BamHI and EcoRI are restriction enzymes used for digestion of BACs. Each left line is the 166 kb KA1 BAC and right one is the modified KA1 BAC inserted the htTA gene (see 2.3.1.4 in part of results).

KA1 gene containing 148 kb upstream of translational start codon ATG, 132 bp ATG harbouring exon (exon 1), 18 kb downstream of ATG exon, and exon0 (Figure 9A and C). The existence of the 27 bp of untranslated exon (exon0) on the BACs was also confirmed by Southern blot analysis with the 27 nucleotides (Figure 9D).

2.3.1.3. Construction of targeting vector for homologous recombination of the KA1 BAC

The 166 kb KA1 BAC clones were modified to express the htTA and prokaryotic tTA gene under the KA1 promoter control through homologous recombination. To insert the htTA and prokaryotic tTA respectively into the exon1 harbouring the translational start of the 166 kb KA1 BAC, two amplified recombinogenic arms (A; 630 bp, B; 560 bp) by PCR were on both sides of the targeting cassette, which contains the htTA or tTA gene, a polyadenylation sequence of human growth hormone gene and a selection marker gene (Kanamycine resistant gene, KM) (Figure 10). To eliminate the selection marker gene before microinjection, the KM gene is flanked by FRT (Flp recombinase target) sites, because this selection marker is needed only in *E. coli* during homologous recombination and the BAC plasmid (pBeloBAC11) harbours already one loxP site.

2.3.1.4. Modification of KA1 BAC

To modify BACs containing the KA1 gene to express the tTA gene under the KA1 promoter control, a temperature-sensitive shuttle vector based system was used for homologous recombination in *E. coli*, because BACs are propagated in the recombination deficient *E. coli* host cell (recA$^-$), DH10B. There is often failure in obtaining intact size of BACs during a transfer of BACs to the other strain, which can be used for homologous recombination. To overcome the deficit of recombination in BAC host cells, the *E. coli* recA was introduced into BAC host cell DH10B (recA$^-$) via the temperature-sensitive shuttle vector (pSV1.recA), which replicates at the permissive temperature (30^{o}C) but is lost at the restrictive temperature (43^{o}C). Thereby it is possible to modify BACs directly in the recombination deficient host cell and to return to a recombination deficient condition for the stability of the modified BACs (Yang X.W. et al. 1997) (Figure 11).

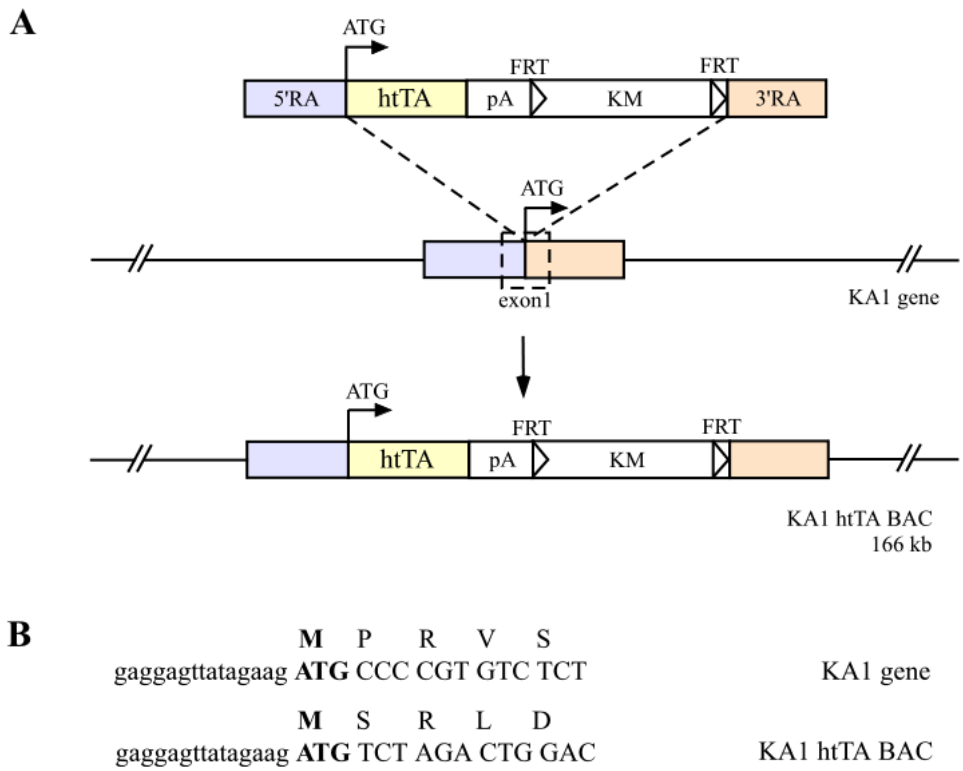

Figure 10. Generation of BAC carrying the KA1 gene to express the htTA under a control of the mouse KA1 promoter (illustrated only in case of the htTA but for the prokaryotic tTA via the exact same way). The htTA gene was inserted into the exon1 harbouring translation start codon of the KA1 gene on the 166 kb BAC by homologous recombination. (A) The targeted htTA cassette and locus of insertion on the KA1 BAC. Open lined box on the KA1 gene indicates the 132 bp exon1 containing translational start codon ATG; RA, recombinogenic arm amplified by PCR; htTA, the humanised tTA gene; pA, polyadenylation signal of human growth hormone; KM, Kanamycine resistance gene; FRT, Flp recombinase target site. (B) Small letters show the 5' untranslated sequences and capital letters are codons with single letter amino acids for the KA1 and the htTA from modified KA1 BAC.

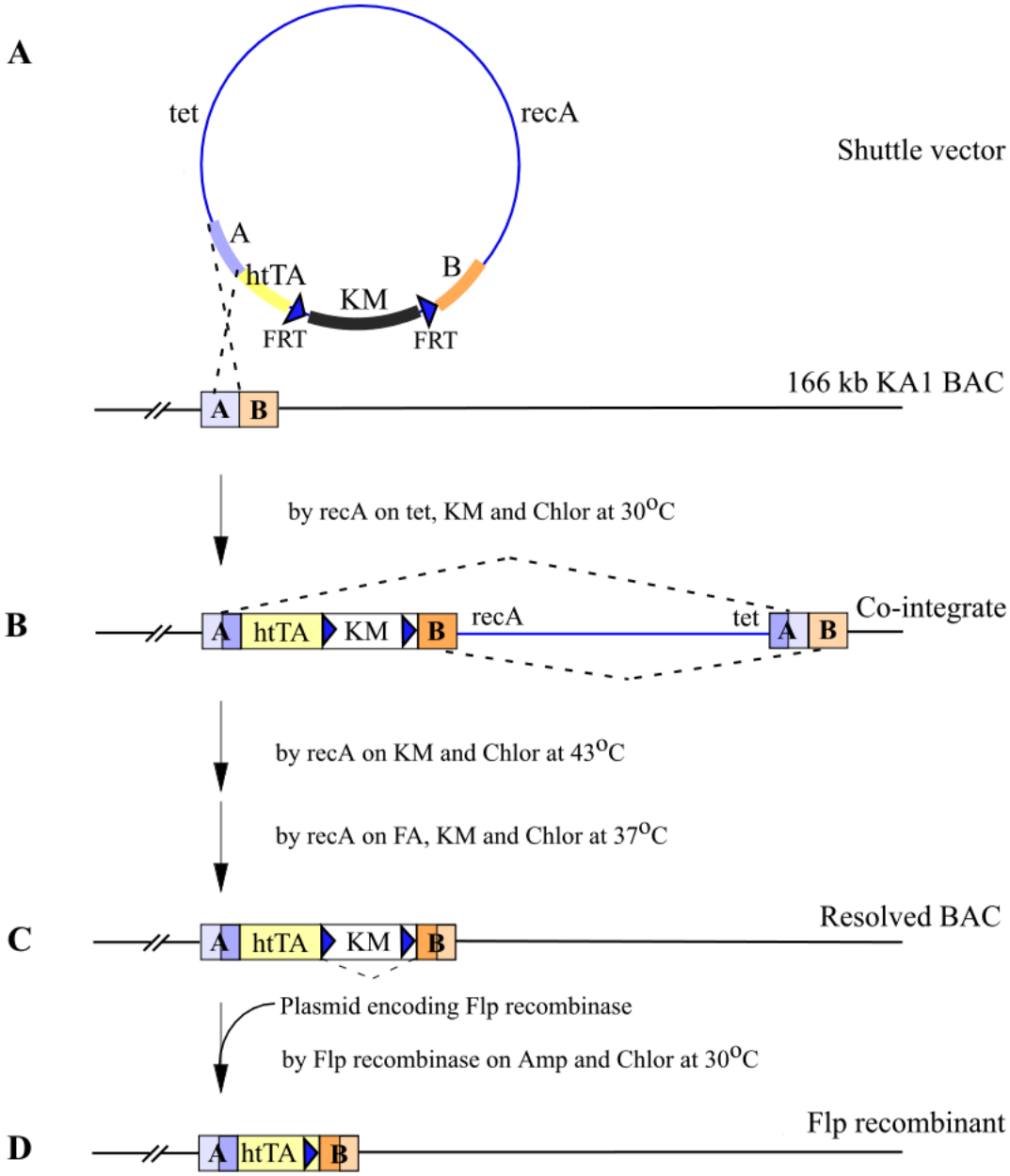

Figure 11. The strategy to modify the 166 kb KA1 BAC. (A) Targeting cassette is integrated into the 166 kb KA1 BAC by recA via shuttle vector (pSV1.recA-htTA) based on temperature-sensitive origin (illustrated only in case of the htTA but for the prokaryotic tTA via the exact same way). (B) Cointegrate can be formed by homologous recombination through either A or B (only A case is illustrated) at 30°C. (C) Second recombination through B and subsequent temperature- and antibiotic-selection can produce resolved BAC by recA integrated into BAC. (D) The selection marker flanked with FRT can be eliminated by Flp recombinase. The Flp recombinase plasmid (pMAK-705FLP.amp) should be transformed into the cells containing the resolved BAC and incubated at 30°C. (Amp, ampicilin; Chlor, chloramphenicol; FA, fusaric acid; KM, kanamycine; tet, tetracycline; and FRT, Flp recombinase target site)

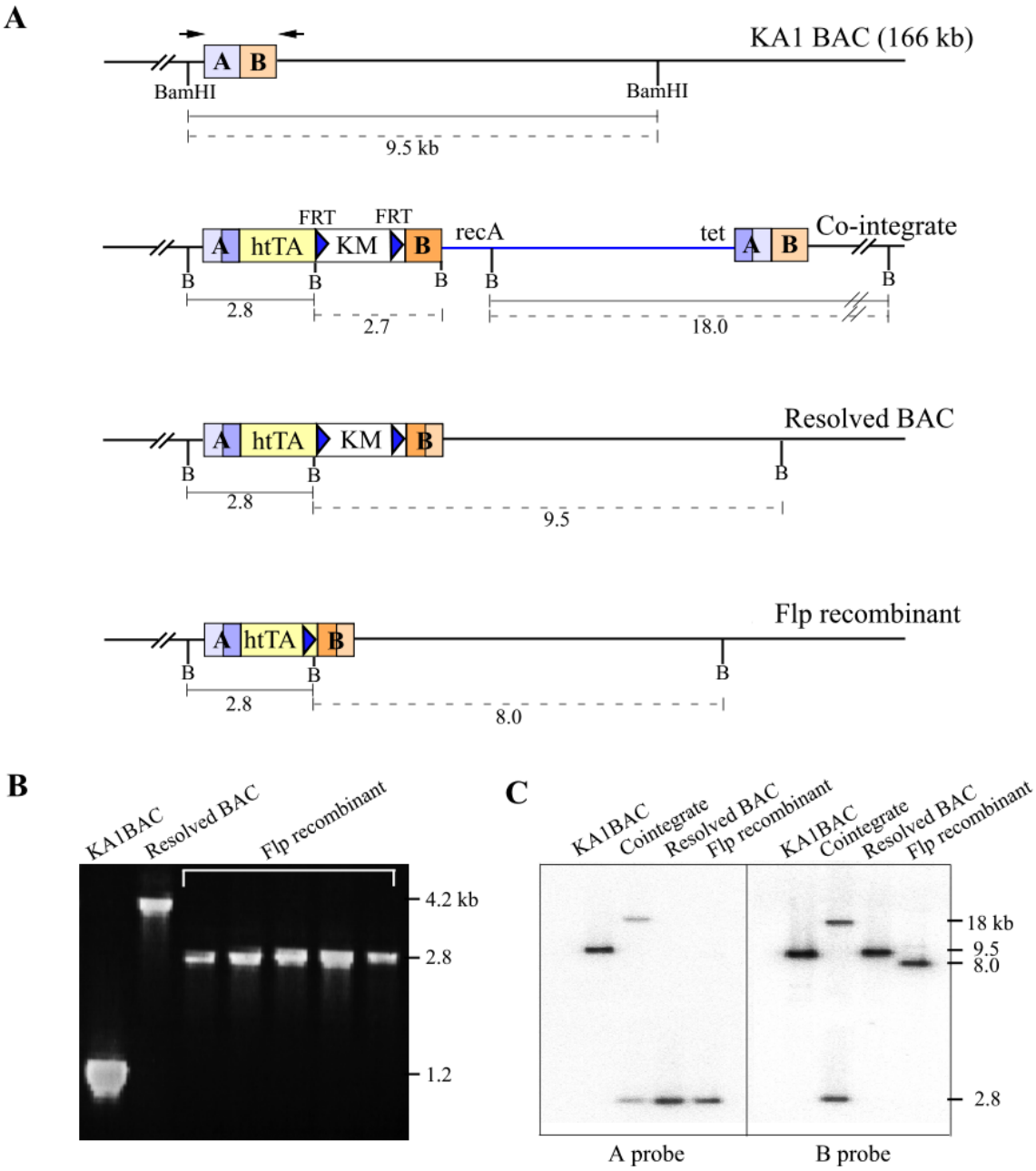

Figure 12. Generation of the modified KA1.htTA BAC. (A) Schematic representation of expected BamHI-digested fragments in the KA1 BAC, cointegrate, resolved BAC and Flp recombinant by southern analysis with recombinogenic arm A probe (thin line) and recombinogenic arm B probe (open line) Two arrows indicate outside primers for colony PCR; FRT, Flp recombinase target sites; B, BamHI restriction site. (B) Conlony PCR of the KA1 BAC resolved BAC and Flp recombinants with outside primers. All 5 Flp recombinant, which were randomly picked show correct PCR fragment. (C) Southern blot analysis of the KA1 BAC, cointegrate, resolved BAC and Flp recombinant. BamHI-digested BAC DNAs of each step were hybridized with recombinogenic arm A probe (630 bp) and recombinogenic arm B probe (560 bp), respectively.

(1) Shuttle vector and first recombination

The htTA and tTA gene targeting cassettes were subcloned into a temperature-sensitive shuttle vector (pSV1.recA.NotI) (Figure 11A). The targeting cassettes containing shuttle vectors (pSV1.recA-htTA and –tTA, respectively) were then transformed into KA1 BAC host cells. The transformants were selected by tetracycline resistance (tet, carried by pSV1.recA,NotI), kanamycine (KM, carried by the targeting cassettes) and chloramphenicol (Chlor, carried by the KA1 BAC vector). The first homologous recombination occurred by recA expressed via the pSV1.recA between the shuttle vector and KA1 BAC through either the 5' recombinogenic arm (A) or 3' recombinogenic arm (B) in cells growing at 30°C on plates containing tetracycline, kanamycine and chloramphenicol. Thereby this whole part of the shuttle vector as well as the targeting cassette were integrated into the KA1 BAC, termed co-integration (Figure 11B). Three of 24 clones were determined by southern blot analysis as correct cointegrates that have occurred through A.

(2) Second recombination of the cointegrates

Correct cointegrates underwent the second homologous recombination in cells growing on kanamycine and chloramphenicol plates at 43°C, called resolution (Figure 11C). This recombination event should result from the recA carried by only integrated shuttle vector, either into the KA1 BACs or bacterial chromosome, because the temperature of 43°C is nonpermissive for replication of the shuttle vector. The resolved BACs were subsequently selected by growing on kanamycine, chloramphenicol, and fusaric acid (FA) plates at 37°C, resulting from the fusaric acid causing negative selection for the loss of tet resistance (Maloy et al. 1981). Through the second homologous recombination and subsequent selections, the recA on the KA1 BAC was eliminated and thus a recombination deficient host cell condition obtained for the resolved KA1.htTA.KM BACs. During cloning of the targeting cassette into the shuttle vector and second recombination, the selection marker helped easily to get correct clones. Otherwise there are two possible recombinants after the second recombination, such as a resolved BAC and original BAC because of two-repeated recombinogenic arms. Almost 100 % clones, however, were correctly recombined with a selection of kanamycine. Colony PCR and southern blot analysis identified resolved BACs (Figure 12).

(3) Flp recombination of the resolved KA1-htTA/tTA.KM BAC

To eliminate the FRT-flanked kanamycine resistant gene, the Flp recombinase plasmid (pMAK-705FLP.amp), which is based on the pSC101 with temperature

sensitive replication origin to permit simple elimination of the plasmid after site-specific recombination (Hashimoto-Gotoh and Sekiguchi 1997), was transformed into the DH10B cell containing the resolved BAC (Figure 11D). The cells transformed with pMAK-705FLP.amp were incubated at 30°C and subsequently at 37°C on ampiciline and chloramphenicol plates. After Flp recombination any colony grew neither in the presence of kanamycine nor ampiciline, and 100 % site-specifically recombined. The finally modified BACs were also verified by colony PCR, southern blot and sequencing (figure 12). Pulsed-field electrophoresis gel (PFEG) and restriction maps with several edonucleases and southern blot pattern revealed no change of BACs after the final modification. By NotI digestion the modified KA1 BACs were released from the BAC plasmid (pBeloBAC11) and additionally lost 13 kb of 5' regulatory region via endogenous NotI site. However, the NotI-linearized KA1.htTA BACs and KA1.tTA BACs still contain 153 kb of the KA1 gene, i.e. 135 kb 5' regulatory region upstream of exon1 (Figure 9A). Thus NotI-linearized KA1.htTA BACs and KA1.tTA BACs, respectively, were purified by Sepharose CL-4B gel fraction and injected into the male pronuclei of fertilized mouse oocytes.

2.3.1.5. Analysis of KA1.htTA and KA1.tTA founders

By tail biopsy of genomic PCR, the existence of htTA and tTA transgenes in potential founder animals was assessed: 7 founders out of 49 pups for KA1.htTA and 8 founders of 51 for KA1.tTA. To determine if the intact BACs had been integrated into the mouse genome, one of the BAC terminals was amplified by PCR, because BACs linearized with NotI contain some vector sequence. Incomplete integration was found in one of 15 founders, including KA1.htTA and tTA. By unidentified reasons some of those founders lacked transgene transmission or the ability to reproduce. To visualize expression pattern and activity of the htTA and tTA in these transgenic mice, the founders were bred with lacZ indicator lines (M3 and MNL), which contain a tTA-dependent minigene encoding the NMDA receptor subunit NR1 and lacZ in a bidirectional module (Jerecic et al. 1999) (Figure 13).
The double-transgenic animals of $Tg^{KA1.htTA/MNL\ or\ M3}$ and $Tg^{KA1.tTA/MNL\ or\ M3}$ were analyzed by staining for β-galactosidase activity on brain sections at ages postnatal day 7 (P7) and P42. Unfortunately, no blue staining was observed in brain sections in all animals even after 30 hours incubation for β-galactosidase activity. Nevertheless the existence of the htTA or tTA mRNA under the control of the KA1 promoter in brain was verified by reverse transcriptase-PCR (RT-PCR). 5 of 6 founders of KA1.htTA lines expressed htTA mRNA in mutant brains (figure14). For KA1.tTA lines only 1 out of 4 founders proved RT-PCR positive. With most of analyzed founders expressing RT-PCR detectable levels of tTA, the failure in induction of the

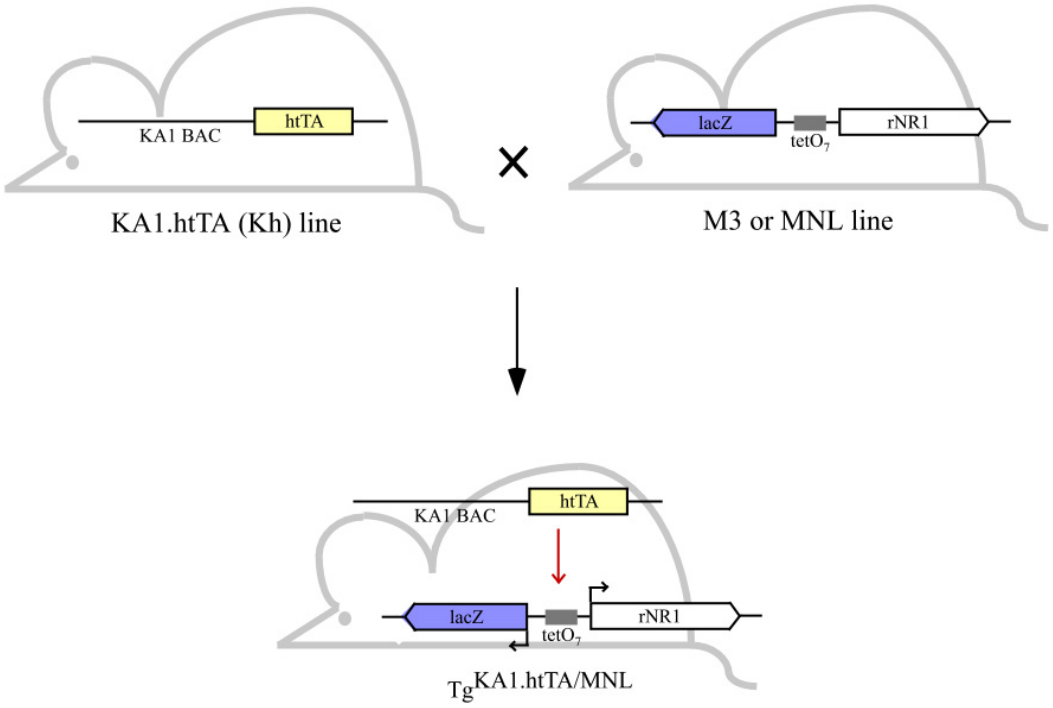

Figure 13. Genetic cross to visualize the activity and expression pattern in the KA1-htTA (or KA1-tTA) transgenic mice. The KA1-htTA lines were bred with the lacZ indicator line (M3 or MNL) containing tTA-dependent NR1 and lacZ gene in a bidirectional module. In double transgenic animals of $Tg^{KA1\text{-}htTA/MNL\ or\ M3}$, the expressed htTA can activate expression of lacZ gene and then activity and expression pattern of the htTA can be visualized by X-gal staining: tetO7, seven tet repressor binding sites; rNR1, rat NMDA receptor subunit 1.

tTA-responsive gene in these mice might be explained by insufficient amount of tTA expression under the KA1 promoter driving from BACs. For the functional activity of tTA, a large supply of tTA seems to be required in brain and expression via BACs containing the KA1 promoter might not be strong enough. However, in terms of frequency of expression of the htTA and tTA gene in transgenic mice, the results of RT-PCR demonstrated that the utility of humanized tTA for transgenic mice is much more promising than that of the prokaryotic tTA.

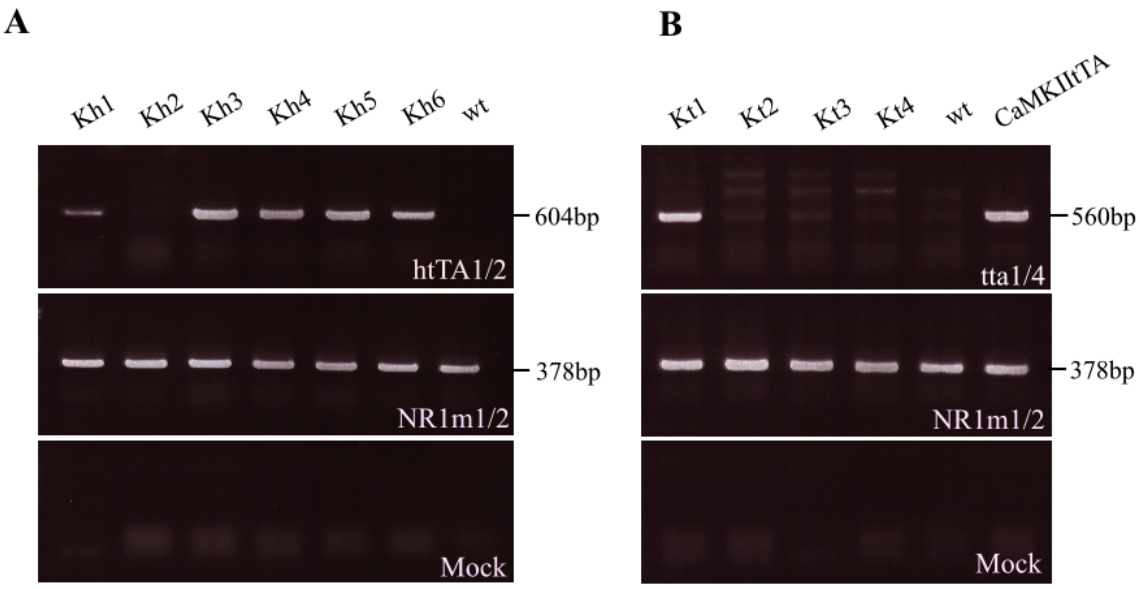

Figure 14. Reverse transcriptase-PCR (RT-PCR) verify the existence of the htTA and tTA mRNA in transgenic lines. (A) Total RNAs from brains of KA1-htTA transgenic lines (Kh1-6) and wild type were amplified with htTA1/2 sitting on the htTA gene and NR1m1/2 sitting on different exons of the NR1 gene as a control. Mock was performed without reverse transcriptase. (B) RT-PCR of the KA1-tTA lines (Kt1-4), wild type, and $Tg^{CaMKIItTA}$ (Mayford et al. 1996) with tta1/4 for the tTA gene and with NR1m1/2.

2.3.2.Generation of CaMKII-NR2C.htTA transgenic mice

We next attempted to generate transgenic mice expressing the htTA by an alternative approach. Neurospecific gene expression can generally be achieved using a neuron-specific promoter such as the CaMKIIα promoter (Mayford, M. et al. 1996), the neuron–specific enolase promoter (Peel et al. 1997) or YAC/BAC containing KA1 promoter (Kask et al. 2000). An alternative approach was to repress ubiquitous expression using a negative regulatory element; neuron-restrictive silencer element (NRSE). The regionally restricted expression of the htTA in brain could be obtained by using the 8.5 kb fragment of CaMKII promoter that is transcriptionally suppressed by the 1.0 kb fragment of NR2C including NRSE to prevent widespread expression of the htTA in brain.

2.3.2.1. Characterization of the NR2C gene

To increase the knowledge of how the NR2C gene expression is controlled with a developmental and cell environmental specificity, the gene structure has been characterized and variable fragments up and downstream of the transcriptional start site were used to direct reporter gene in neuronal/non-neuronal cells and transgenic mice (Suchanek et al. 1995 and 1997). The NR2C subunit is mainly expressed in the cerebellar granule cells starting from the second week of postnatal life (Monyer et al. 1992). The 5' untranslated region of the NR2C gene (*Grin2c*) includes three exons (exon 1-3) with two transcriptional start sites at –772 and –754 bp from the translational state site: 254 bp of exon 1, 172 bp of exon 2, and 235 bp of exon 3, interrupted by introns of 398 bp and 106 bp respectively (Figure 15). It was demonstrated that the 0.4 kb segment upstream of the transcriptional start site of the NR2C gene (*Grin2c*) has a general promoter activity in transgenic mice determined by the β-galactosidase expression pattern under the control of this 0.4 kb fragment of NR2C gene. On the other hand, the 1.0 kb segment (exons 1-3) downstream of the transcriptional start site directed the specific expression in cerebellar granule cells with weaker additional expression in other brain regions, i.e. dentate gyrus, olfactory bulb and cortex in transgenic mice. This 1.0 kb region of exon 1, 2, 3 of the NR2C gene seems to negatively regulate the transcription in NR2C negative cells for NR2C-specific expression. The 1.0 kb region contains a neuron-restrictive silencer element (NRSE)-like sequence near the 5' end of exon 1. This 21 bp conserved negative transcriptional element (NRSE) has been identified in many neuronal genes such as N-methyl-D-aspatate receptor (NMDA1, NR2C), brain-derived neurotrophic factor (BDNF), γ-aminobutyric acid (GABA), nicotinic acetylcholine receptor (nACh) and

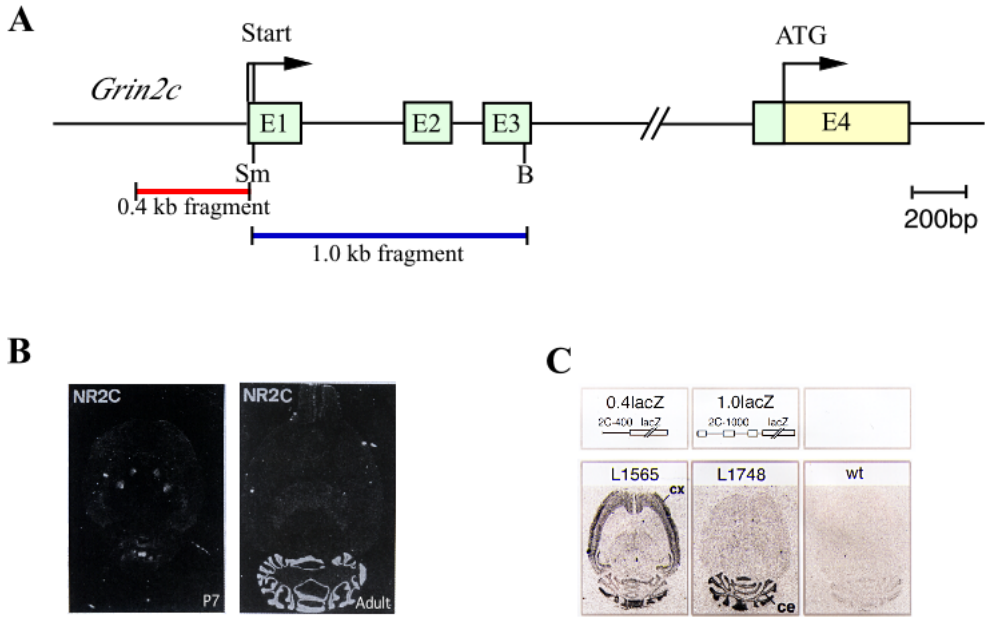

Figure 15. The proximal promoter region and silencer region of the NMDA receptor subunit 2C. (A) The gene structure of proximal promoter region of the *Grin2c*. Left arrow indicates two transcription initiation sites, right arrow translational start site. Bright green boxes show untranlslational exons (E1, 2 and 3), yellow translational exon (E4). The distance between exon3 and 4 is reported approximately 6 kb. The 0.4 kb promoter fragment characterized as a basal promoter and the 1.0 kb silencer fragment are indicated by red and blue line, respectively. (B) In situ hybridization for the NR2C transcripts in horizontal slices of rat brain at P7 (Left) and adult (Right). (C) Schematic representation of transgenes (upper panel). *Left*, the 0.4 kb of NR2C promoter upstream of first exon and lacZ gene; *Right*, 1.0 kb of exon 1-3 and lacZ. The expression of β-galactosidase transcripts in transgenic mice determined by in situ hybridization (down panel).(based on Suchanek B. et al. 1997): B, BamHI site; Sm, SmaI restriction site; ce, cerebellum; cx, cortex.

so on and is located in 5' untranslated regions (5' UTR) or in intragenic positions (Schoenherr et al. 1996) (Table2). The 1.0 kb fragment of NR2C gene might be contribute to tissue specificity through selective suppression of the transcription and thus be useful for the generation of transgenic mice expressing the htTA in a spatial manner.

Table 2. Genes with NRSE-like sequences

Gene	Sequence comparison	Binding activity*	Silencing activity*	Location
Consensus	TTCAGCACCACGGACAGCGCC			
Neuronal genes				
Rat SCG10	----------G--T---	+	+	5′-Reg
Rat type II sodium channel	-----A----------A--	+		5′-Reg
Human synapsin	---------G-------T---	+	+	5′-Reg
Rat BDNF	---------TT------A---	+		Intron
Rat NMDA rec. 1	---------T--------AT-	+	+	5′-UTR
Human nicotinic ACh rec. β2	----------	+	+	5′-UTR
Chicken β4-tubulin	---------G----------	+	+	Intron
Chicken middle neurofilament	----------T--	+		5′-Reg
Human glycine rec.	----------G----T-	+	+	5′-UTR
Rat glycine rec.	---------T-A------T--	+		5′-UTR
Rat synaptophysin	-C-------GT------A---	+	+	Intron
Human L1	----------G--------AA	+		Intron
Rat atrial natriuretic peptide	----------ACG-	+		3′-UTR
Mouse calbindin	AG-------G----------	+		5′-UTR
Rat GABA-A rec. δ subunit	---------GA----G---GA	+	+	Intron
Rat nicotinic ACh rec. α7	AG----G--G---C---A---	−	−	5′-UTR
Mouse P-Lim	---------G----------			5′-UTR
Mouse Hes-3	GG----------			Coding
Human CRF	---------G----------			Intron
Human olfactory rec.	G--------CA----------			Coding
Mouse synaptotagmin IV	---------T-------A--A			5′-UTR
Mouse AMPA rec.	--T------G---------T			5′-Reg
Rat VGF	--------GCT----------			5′-UTR
Rat proenkephalin	--------AC----G--G---			Intron
Mouse NMDA rec.	**--GC-----CG-----CA---**		**+**	5′-UTR
Nonneuronal genes				
Rat APRT	G--------C-------G---	+		Intron
Sheep keratin	A----------G-	+	+	5′-Reg
Mouse skeletal actin	GG----------C-------	+		3′-Reg
Bovine P450-11β	---------TA------A-G-	+	+	Jxn
Human P450-11β	---------TA------AAG-	+		Jxn
Human T-cell rec. β	G------A-CT------A---	−	−	Coding
Human myosin light chain	C--------CAT-----T---	−	−	Coding
Mouse macrophage protein	CC-------T-A-C-------	−	−	Coding
Rat troponin T	------C--TA------C---			Intron
Rat somatostatin trans. factor	---------T-------A--A			5′-UTR

Table 2. All genes with two or fewer mismatches to the composite NRSE used in the data base search are listed. Selected genes with three or greater mismatches, for which NRSF binding was experimentally determined, are listed as well. The NRSE-like sequences are compared to the revised consensus NRSE. Derivations shown are derived from comparison to this revised consensus and not to the composite NRSE used in the original data base search. The NRSE-like sequence of the NR2C is added in red: Rec., receptor; BDNF, brain-derived neurotrophic factor; NMDA, N-methyl-D-aspatate; Ach, acetylcholine; GABA, γ-aminibutyric acid; CRF, corticotropin-releasing factor; AMPA, α-amino-3-hydroxy-5methyl-4-isoxazole-propionic acid; VGF, vascular endothelial growth factor, APRT; adenine phosphoribosyltransferase; trans., transactivating; Reg, 5´´-regulatory region; 5´- or 3´-UTR, 5´- or 3´-untranslated region; Jxn, intron/exon junction.

* A blank space indicates that the sequence has not been tested for the activity.

2.3.2.2. Construction of the minigene of CNhtTA and sCNhtTA

To generate transgenic mice expressing the htTA in a region-restrictive fashion in brain, the forebrain-specific CaMKIIα promoter was fused with the NR2C gene silencer region (exons 1-3). The chimeric promoter comprises the CaMKIIα promoter, the tripatite leader sequence, and the 1.0 kb fragment from exon 1 to exon 3 of the NR2C subunit gene as transcriptional negative element (Figure 16).

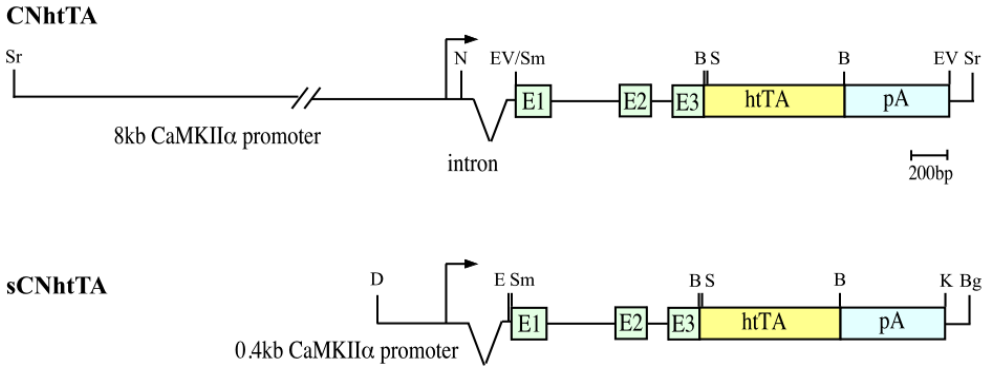

Figure 16. Constructs of 0.8kbCaMKIIpromoter-NR2C silencer-htTA (CNhtTA) and small 0.4kbCaMKIIpromoter-NR2C silencer-htTA (sCNhtTA) minigenes. The CaMKII promoter is fused with 1.0 kb silencer of the NR2C (exon1-3 in green boxes). Tripatite intron is indicated by triangle. The htTA gene with polyadenylation signal of human growth hormone (pA) is followed next to the chimeric promoter. Several restriction sites, which were used for cloning and releasing the minigene from cloning vector; Sr, SrfI; N, NotI; EV, EcoRV; Sm, SmaI; B, BamHI; S, SalI; D, DraIII; E, EcoRI; K, KpnI; Bg, BglII.

The CaMKIIα promoter consists approximately 8.5 kb of genomic DNA upstream and 89 bp downstream of the transcription initiation site of the mouse CaMKIIα gene (Mayford et al. 1996). The adenovirus tripatite leader sequence, which is known to enhance RNA stability and efficiency of translation (Choi et al. 1991 and Sheay et al.1993), follows next. The 1.0 kb (exon1-3) untranslated fragment of mouse NR2C gene was added downstream of the CaMKIIα promoter and the synthetic intron sequence. The two transcriptional start sites on the first exon of the NR2C gene itself and the splicing donor of the end of the third exon were removed on the chimeric promoter, to abolish unfavored additional transcriptional events. Alternatively instead of the 8.5 kb CaMKIIα promoter fragment, we also used a 0.4 kb fragment of the CaMKIIα promoter because we experienced that the 0.4 kb small fragment of the CaMKIIα promoter region seems to include enough elements to control the forebrain-

specific expression of a transgene in transgenic mice. To direct the htTA expression under the chimeric promoter, the humanized tTA gene was flanked by the chimeric promoter at the 5' end and by 600 bp of human growth hormone polyadenylation sequence at 3' end. The minigenes 8.5kbCaMKII-NR2C-htTA (CNhtTA) and 0.4kbCaMKII-NR2C-htTA (sCNhtTA) were purified by sucrose gradient and injected into the male pronuclei of fertilized mouse oocytes, respectively.

2.3.2.3. Analysis of founders of CNhtTA and sCNhtTA

12 founders out of 45 pups of CNhtTA and 14 founders out of 53 pups of sCNhtTA were determined by tail genomic PCR. Of those, some lacked transgene transmission or reproduction ability without definite reason. To assess the regional pattern of the humanized tTA activity under control of the chimeric promoter, transgenic lines were crossed to the lacZ reporter line (MNL) or lacZ/GFP line (G9) (Krestel et al. 2001), and brain sections of double transgenic mice were analyzed by β-galactosidase-mediated X-gal staining and immunocytochemistry with antibodies against β-galactosidase. Immunocytochemistry permits more sensitive analysis and enabled us to specifically identify the expressing cells, because X-gal staining sometimes shows diffuse blue signals. Among 8 transgenic lines of both sCNhtTA and CNhtTA, which were analyzed so far, three lines were identified as the functional tTA-expressing mice. The X-gal stain of sagittal sections showed that the expression and activity of the htTA in the double transgenic animal ($Tg^{sCN1/MNL}$) were highly specific to the granule cells of the dentate gyrus at P28 (Figure 17). In the dentate gyrus on the 100μm thick sections, β-galactosidase expression seemed to be in all granule cells but X-gal staining and nuclear counterstaining on the 15μm thick brain section, which is believed to contain roughly one cell layer, should be performed for the study of a mosaic pattern in sCN1 line. The blue X-gal signals were detected in cell nuclei because of the nuclear localization signal-containing lacZ gene in the MNL responder line. There were no detectable X-gal positive signals in striatum, cortex, thalamus, cerebellum and brainstem of both lines $Tg^{sCN1/MNL}$. By breeding with $Tg^{CaMKIItTA}$, the MNL line was determined as an efficient responder showing the expression of β-galactosidase in striatum, cortex, CA1, dentate gyrus, and olfactory bulb of $Tg^{CaMKIItTA/MNL}$.

In the CN10 line ($Tg^{CN10/MNL}$), the htTA was expressed and active in the pyramidal cells of CA1 and CA2, the granule cells of dentate gyrus and some olfactory neurons at P50 (Figure 18). Little or no staining was observed in other brain areas, including striatum, thalamus, cerebellum, and brainstem of the CN10 line.

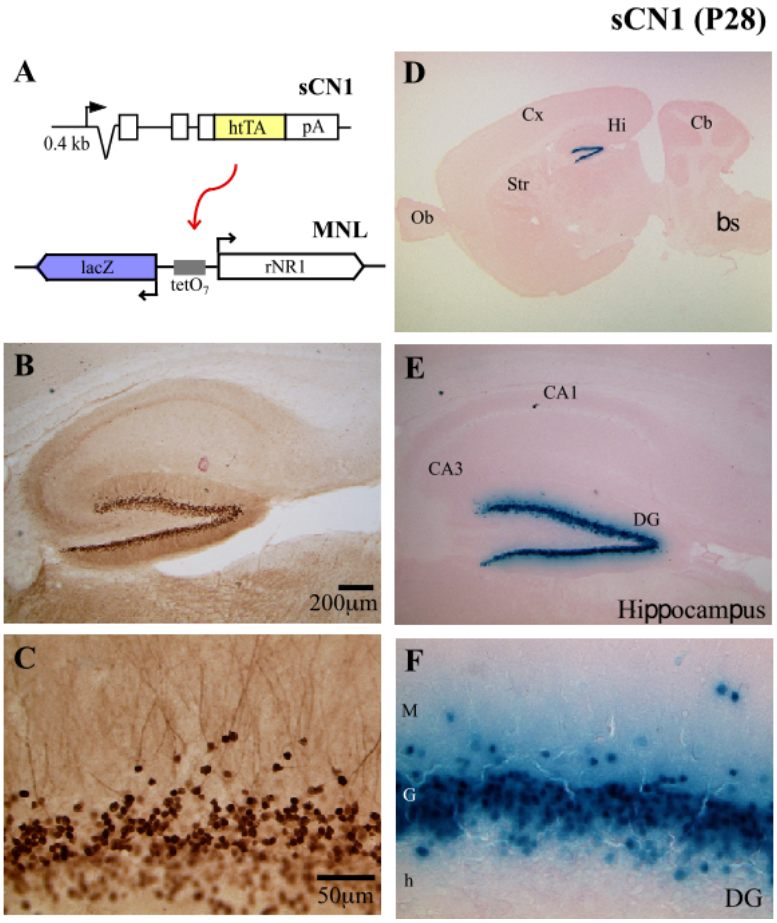

Figure 17. Expression pattern and activity of the htTA in sagittal section of the double transgenic mouse $Tg^{sCN1/MNL}$ at P28. (A) Schematic representation of constructs of the sCNhtTA and indicator line containing a bidirectional minigene (MNL). (B) shows hippocampus stained by immunocytochemistry with β-galactosidase antibody and (E) X-gal staining/eosin counterstaining. (C and F) High magnification of the dentate gyrus showing β-galactosidase activity in all granule cells. (D) Overview of X-gal and eosin-stained sagittal section; Cx, cortex; Hi, hippocampus; Cb, cerebellum; Str, striatum; Ob, olfactory bulb; bs, brain stem; CA1/3, areas of Ammon's horn; DG, dentate gyrus; M, dentate molecular layer; G, stratum granulosum; h, hilus (polymorphic zone of the dentate gyrus).

In case of the CN4 line, the G9 responder line carrying green fluorescent protein (GFP) and β-galactosidase in a bidirectional module was crossed as an indicator mouse. *In vivo* analysis of GFP fluorescence was possible in double transgenic animal ($Tg^{CN4/G9}$) at P1. In head of double transgenic $Tg^{CN4/G9}$ living newborn pup, GFP fluorescence displayed the cortex. By GFP fluorescence, X-gal staining and immunocytochemistry, the activity of htTA in CN4 line was observed in the forebrain

in a pattern somewhat resembling the previously CaMKII tTA at P1, including striatum, olfactory bulb and cortex. (Figure 19).

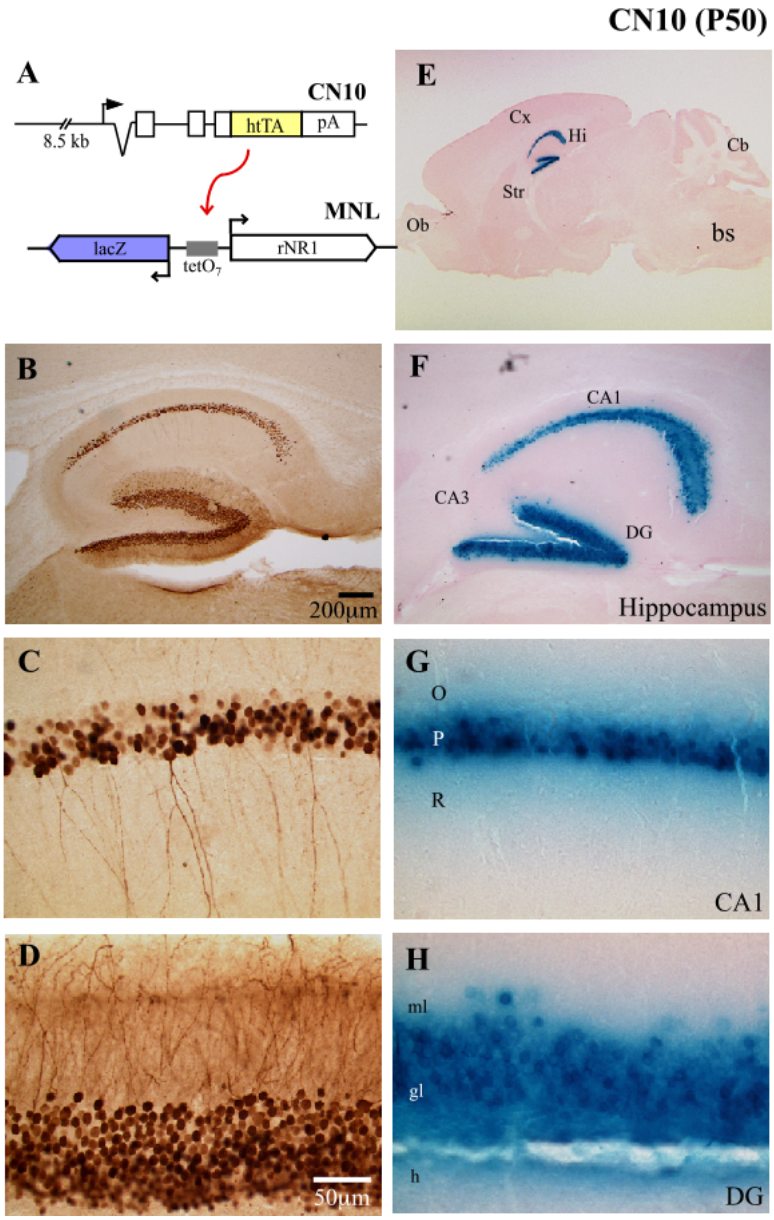

Figure 18. Sagittal sections of the double transgenic mouse $Tg^{CN10/MNL}$ at P50. (A) Schematic drawing of the transgenes of the CNhtTA and bidirectional tTA-responder line MNL. High magnificent observation of hippocampus stained by immunostaining (B, C, and D) and by X-gal (F, G, and H). (B and F) show the htTA activity in the CN10 line in CA1 and DG of hippocampus. The CA1 region (C and G) and DG (D and H) of hippocampus were examined at higher magnification. (E) Overview of the htTA activity in sagittal section determined by X-gal staining and eosin counterstaining: Cx, cortex; Hi, hippocampus; Cb, cerebellum, Str, striatum; Ob, olfactory bulb; bs, brain stem; CA1/3, areas of Ammon's horn; DG, dentate

gyrus; O, stratum oriens; P, striatum pyramidale; R, striatum radiatum; M, dentate molecular layer; G, stratum granulosum; h, hilus (polymorphic zone of the dentate gyrus).

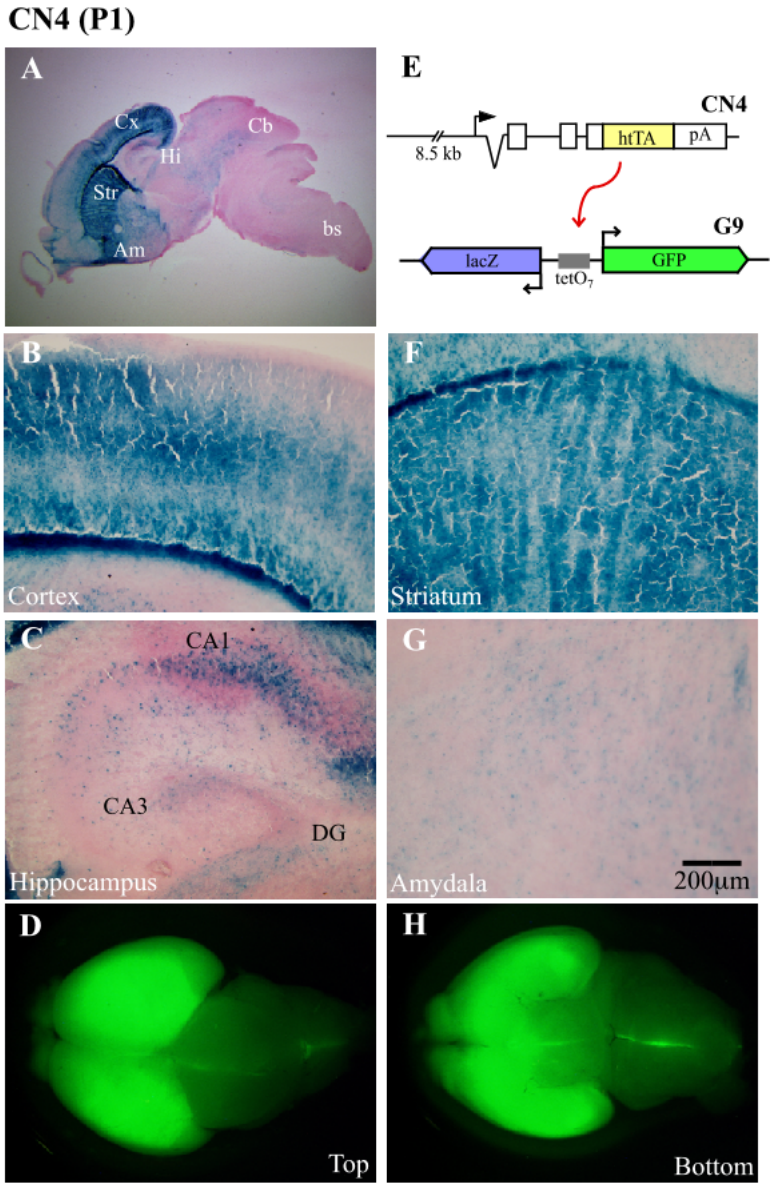

Figure 19. Sagittal sections of the double transgenic mouse $Tg^{CN4/G9}$ at P1 were stained with X-gal and then counter stained with eosin (A, B, C, F, and G). Activity of the htTA was observed in cortex (B), striatum (F) at strong level, and in hippocampus (C), amydala (G) at mild level. (E) Schematic representation of the transgenes of the CNhtTA and tTA-indicator line G9 containing lacZ and GFP in a bidirectional module. (D) and (H) show fluorescence of the unfixed brain via GFP expression.: Cx, cortex; Hi, hippocampus; Cb, cerebellum, Str, striatum; Am, amydala; bs, brain stem; CA1/3, areas of Ammon's horn; DG, dentate gyrus

2.3.2.4. Developmental expression and activity of the htTA in the CN10 line

To investigate the expression pattern and activity of the htTA in the CN10 line during developmental stages, double transgenic animals of $Tg^{CN10/MNL}$ were analyzed at P1, P10, P20 and P50 by β-galactosidase staining and immunocytochemistry (Figure 20).

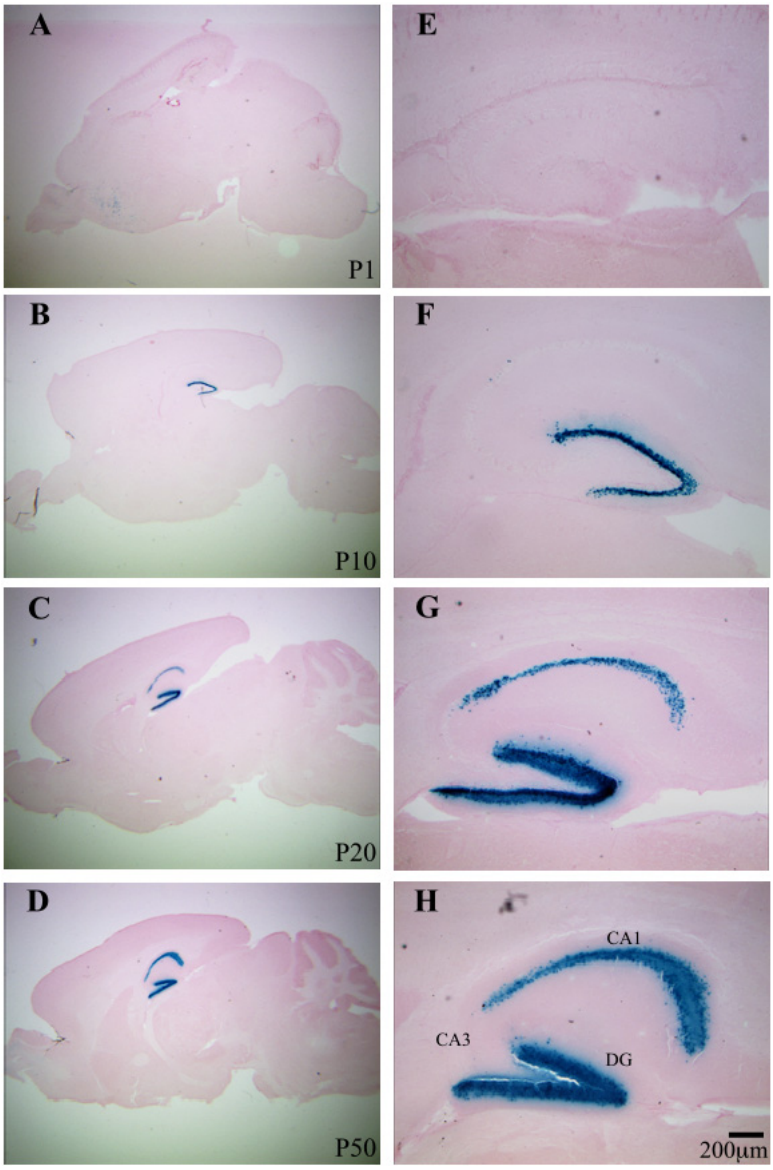

Figure 20. Developmental time window of the htTA activity in $Tg^{CN10/MNL}$. The sagittal sections of brains at P1, P10, P20, and P50 were stained with X-gal and counterstained with eosin. (A)-(D) show overview of the sections at developmental ages. (E)-(H) indicate the hippocampus from the same set of mice.

At P1, no or little X-gal staining was detected in sagittal brain sections of the double transgenic mouse $Tg^{CN10/MNL}$. At P10, the htTA activity was observed in granule cells of dentate gyrus and in CA2 with few numbers of X-gal and immunocytochemistry positive cells. There was no detectable positive signal in pyramidal cells of CA1 at this age. The sagittal sections at P20 and P50 showed similar expression pattern of β-galactosidase, namely in pyramidal cells of CA1 and CA2, and dentate gyrus granule cells. However, the intensity and sensitivity of signals in CA1 at P50 was stronger than at P20 for the same incubation time. On the other hand, in the dentate gyrus there was no difference between these ages.

2.3.2.5. Expression pattern and activity of the htTA in CN10 in detail

To examine the expression and activity of the htTA in CN10 line in the other brain areas, which are undetectable in sagittal sections, the brain of double transgenic $Tg^{CN10/MNL}$ at P42 was serially, coronal dissected and analyzed by X-gal staining and immunocytochemistry (Figure 21). The series of coronal sections were suitable for monitoring cell types with functional tTA activity at macroscopic as well as cellular level in detail (Figure 22 and 23). It was observed that cells with a positive signal by immunocytochemistry were detected in the internal granular layer (ig) of the olfactory bulb, retrosplenial agran cerebral cortex (RSA), caudate putamen (CPu), piriform cortex (Pir) and entorhinal cortex (Ent) as well as CA1/2 and dentate gyrus (DG) of hippocampus. However, by X-gal staining for 30 min of incubation time, intensity and number of positive signals was decreased compared to immunostaining. There were several scattered blue cells in caudate putamen (CPu) with the strongest blue signals in CA1/2 and DG of hippocampus. In the other areas including internal granular layer (ig) of olfactory bulb, retrosplenial agran cerebral cortex (RSA) and striatum, there was no detectable β-galactosidase activity (figure 23-2). Immunocytochemistry allowed a more sensitive measurement for cells containing active htTA.

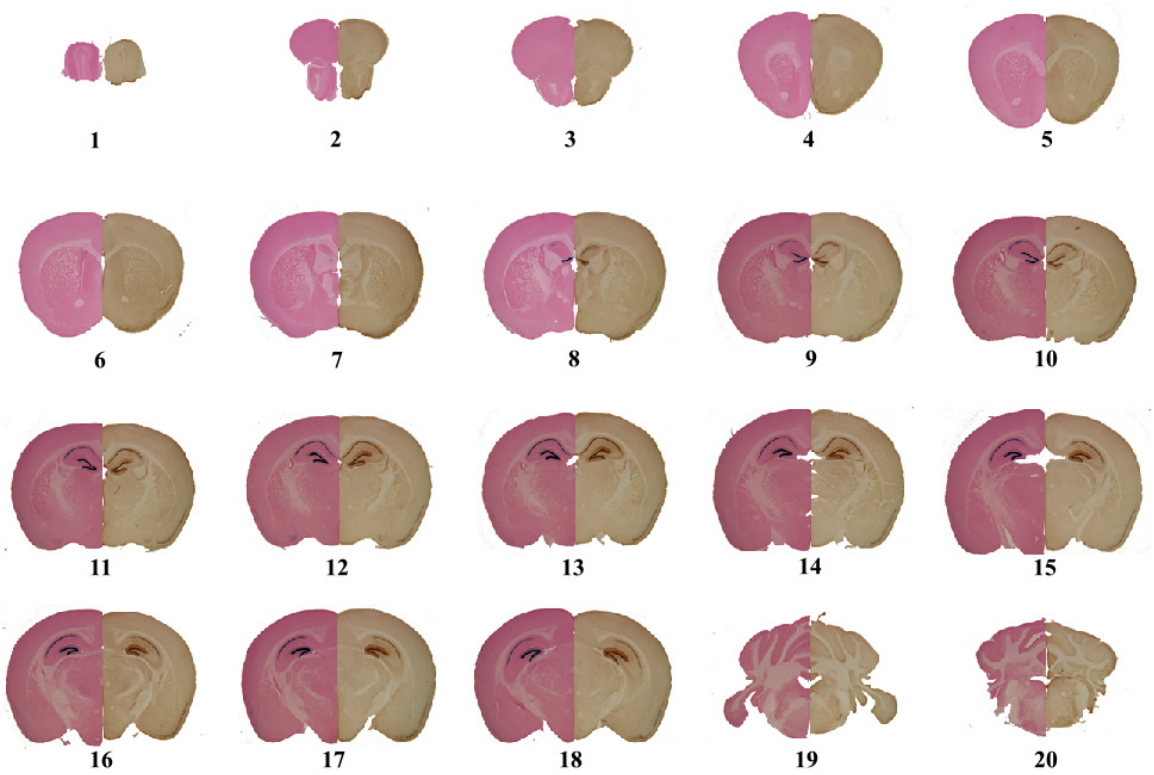

Figure 21. Serial coronal sections of $Tg^{CN10/MNL}$ at P42. Left half of coronal sections were stained with X-gal and counterstained with eosin and right half were performed by immunocytochemistry (ICC) with β-galactosidase antibody. Sections were numbered from rostral to caudal.

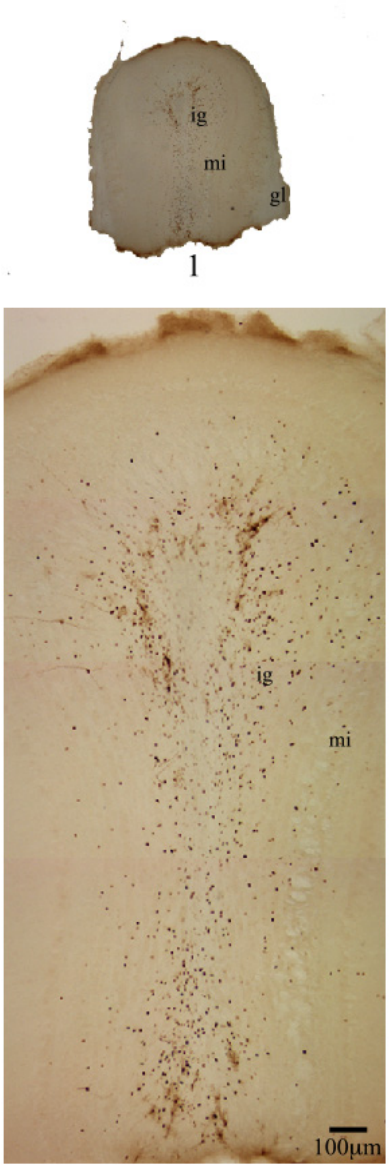

Figure 22. Coronal section of olfactory bulb (serial section number 1 of figure 21) showing some immunostaining in internal granular layer (ig); mi, mitral layer of olfactory bulb; gl, glomerular layer of olfactory bulb.

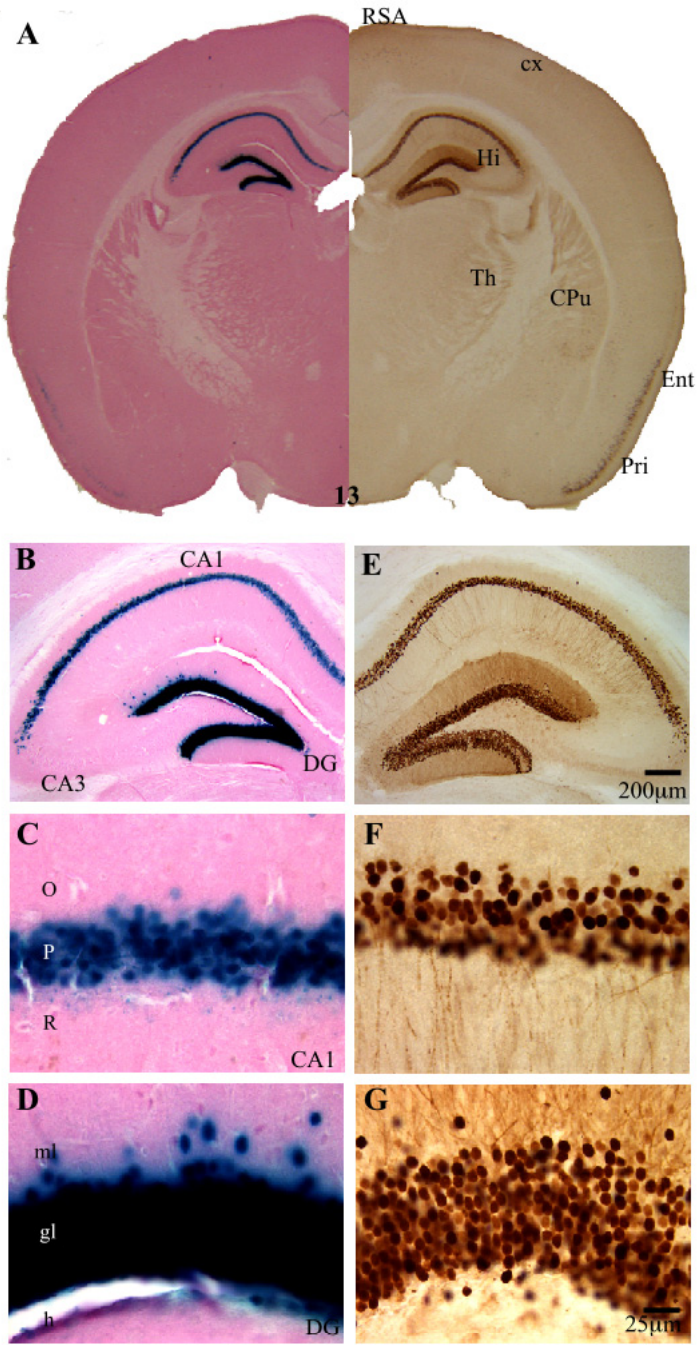

Figure 23-1. High magnification of coronal section number 13 of figure 21. (A) Coronal section. Left panels show X-gal staining, and right immunostaining of the hippocampus. (B and E) give overview of hippocampus. (C and F) High magnification of the CA3 region of hippocampus. (D and G) DG of hippocampus at high magnification. Immunocytochemistry (E-G) permit to identify individual cells without diffused signal, compared to X-gal staining; Cx, cortex; RSA, retrosplenial agran cerebral cortex; CPu, caudate putamen; Pir, piriform cortex; Ent, entorhinal cortex; Th, thalamus; Hi, hippocampus; CA1/3, areas of Ammon's horn; DG, dentate gyrus; O, stratum oriens; P, striatum pyramidale; R, striatum radiatum; M, dentate molecular layer; G, stratum granulosum; h, hilus (polymorphic zone of the dentate gyrus).

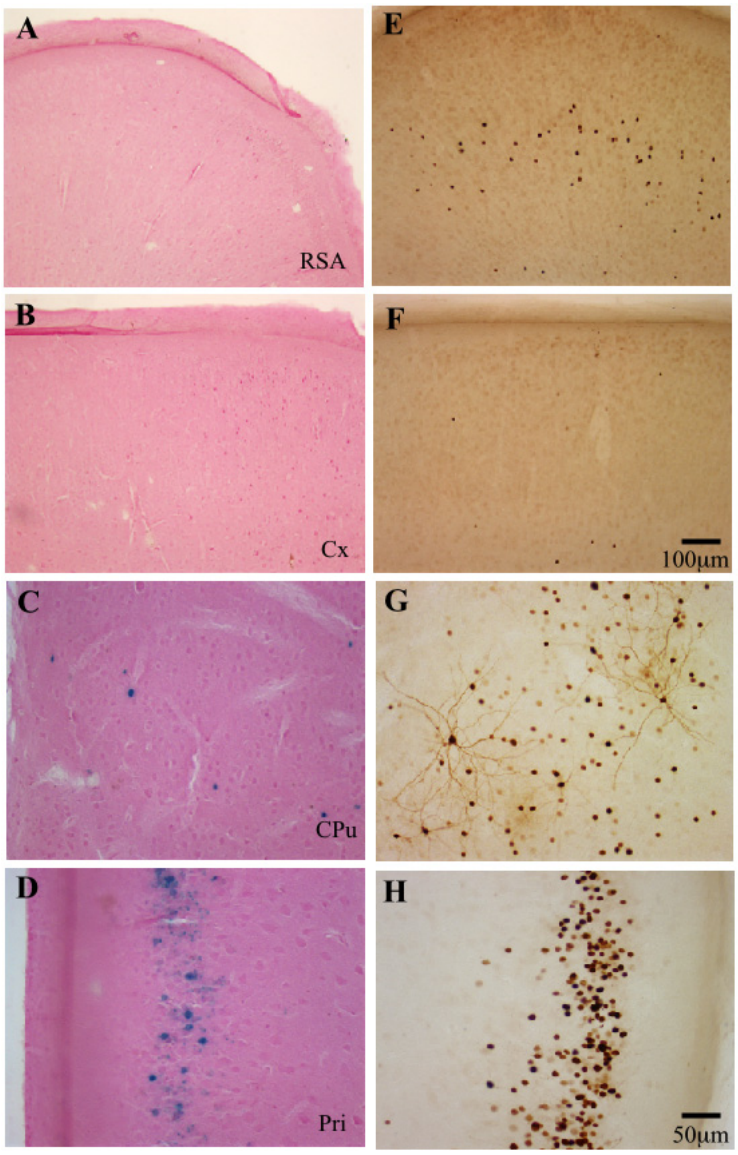

Figure 23-2. High magnification of coronal section number 13 figure 21. Left panels (A-D) show X-gal staining and Right (E-H), immunostaining from coronal section 13. (A and E) In RSA, no X-gal signal was observed contrast to several immunostaining signals. (B and F) There is no detectable positive signal in cortex by immunostaining as well as X-gal. The number of immunostaining positive cells in CPu (G) and Piriform cortex (H) are apparently more than X-gal positive cells (C and D); RSA, retrosplenial agran cerebral cortex; Cx, cortex; CPu, caudate putamen; Pir, piriform cortex.

2.3.2.6. Inducible regulation of gene expression by Dox in CN10

To evaluate suppression and reinduction of the tTA-responsive β-galactosidase expression by doxycycline treatment, double transgenic mice $Tg^{CN10/MNL}$ were raised with doxycycline from conception. Doxycycline should prevent tTA induction, thereby suppressing tTA-responsive gene expression. It was examined that the tTA-inducible β-galactosidase expression in the $Tg^{CN10/MNL}$ at P42 was completely repressed by treatment of doxycycline (50 μg/ml) in drinking water (Figure 24). No X-gal staining positive cells were detectable in CA1 and dentate gyrus of the doxycycline-administered from birth $Tg^{CN10/MNL}$ when compared with the untreated $Tg^{CN10/MNL}$ at P42.

To study a mosaic pattern and reinduction level of β-galactosidase expression, X-gal staining/nuclear counterstaining and immunocytochemistry on the 15 μm thick cryostat sections were performed. In the untreated $Tg^{CN10/MNL}$ at P42, all pyramidal cells of CA1 and granule cells of the dentate gyrus were occupied with X-gal and immunostaining positive signals. The intensity of signals was relatively homogeneous, which could indicate lack of mosaic pattern of the tTA-responsive gene expression. However, the level of expression of β-galactosidase became weaker and the number of β-galactosidase expressing cells was decreased with removal of doxycycline at P21 for 3 weeks, compared to that of the untreated $Tg^{CN10/MNL}$ at P42 (Figure 25). It was found that the number of β-galactosidase staining and immunocytochemistry DAB staining positive cells was decreased in CA1 of $Tg^{CN10/MNL}$ with withdrawal of doxycycline at P21 but not in dentate gyrus, indicating a mosaic pattern. The decreased number and different intensities of β-galactosidase staining and immunocytochemistry staining signals in CA1 indicated incomplete tTA induction at the variable level, perhaps due to storage in other tissues and slow clearance of doxycycline via the blood-brain barrier as previously described (Krestel et al. 2001 and Mack et al.2001).

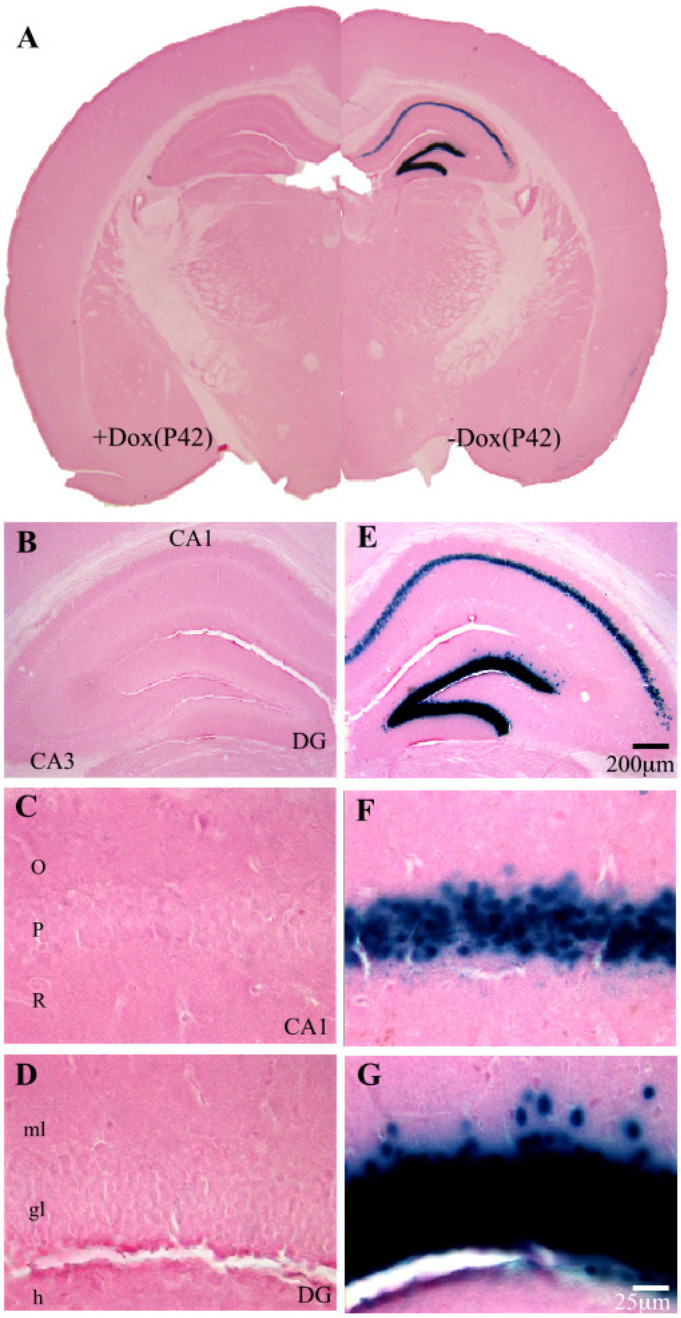

Figure 24. Doxycycline-regulated suppression of the htTA activity in $Tg^{CN10/MNL}$ at P42. (A) Coronal brain sections of $Tg^{CN10/MNL}$, treated by dox from P1 to P42 and untreated, were analyzed by X-gal staining and eosin counter staining at P42. In the hippocampus (B) of the dox-treated $Tg^{CN10/MNL}$, there is no detectable X-gal signal even at high magnification (C and D) compared with in untreated animal (E, F, and G); dox, doxycycline; CA1/3, areas of Ammon's horn; DG, dentate gyrus; O, stratum oriens; P, striatum pyramidale; R, striatum radiatum; M, dentate molecular layer; G, stratum granulosum; h, hilus (polymorphic zone of the dentate gyrus).

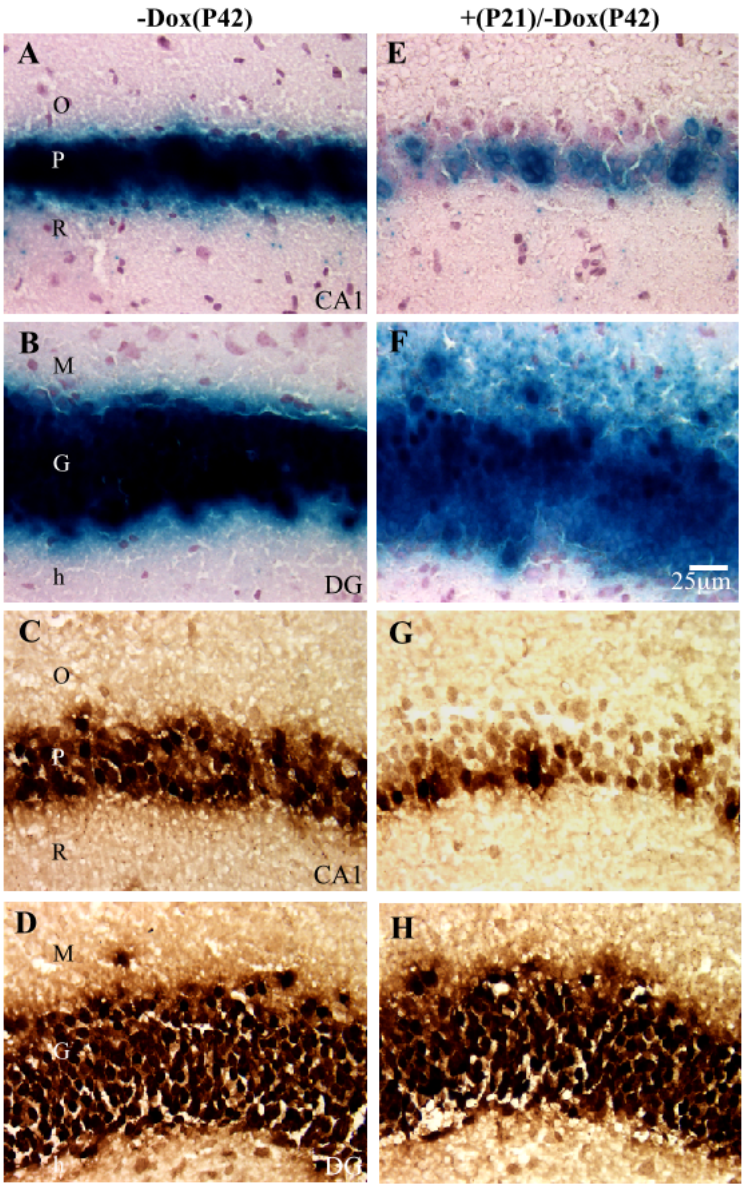

Figure 25. Cryostat sections of $Tg^{CN10/MNL}$ stained with X-gal and neutral red, and by immunocytochemistry. (A-D) Cryostat sections of untreated $Tg^{CN10/MNL}$ at P42. (E-H) That of mice induced at P21 by removal of doxycycline. (A, C, E, and G) reveal that the number of blue X-gal and immunostaining positive pyramidal cells of CA1 region in untreated $Tg^{CN10/MNL}$ at P42 was higher than in mice induced at P21 by removal of doxycycline. Additionally variable intensity of positive signals in CA1 region of P21-reinduced mice was observed (E and G). (B, D, F, and H) X-gal and immuno-stained granule cells of DG in untreated $Tg^{CN10/MNL}$ at P42 and in reinduced mice: dox, doxycycline; CA1, areas of Ammon's horn; DG, dentate gyrus; O, stratum oriens; P, striatum pyramidale; R, striatum radiatum; M, dentate molecular layer; G, stratum granulosum; h, hilus (polymorphic zone of the dentate gyrus).

2.3.2.7. Two different transcripts of the htTA under the chimeric promoter in the CN10 line

The expression pattern and activity of the htTA in the CN10 line is highly restricted in hippocampal CA1 and dentate gyrus. The htTA gene in the CN10 seems to be subregion-specifically directed by the chimeric promoter, especially by the 1.0 kb silencer region. It is interesting how the htTA gene is transcribed and spliced under the chimeric promoter in mice brain of the CN10 line because there are three theoretical introns upstream of the htTA open reading frame (ORF), namely the synthetic intron, two introns between exon 1-2 and exon 2-3 of the 1.0 kb NR2C gene fragment. To assure the splicing pattern of the htTA gene under the control of the chimeric promoter in the transgenic line CN10, hippocampus and forebrain were removed and cDNA were synthesized from isolated hippocampal and forebrain total RNA by 5' RACE (rapid amplification of cDNA end), respectively (Chen 1996).

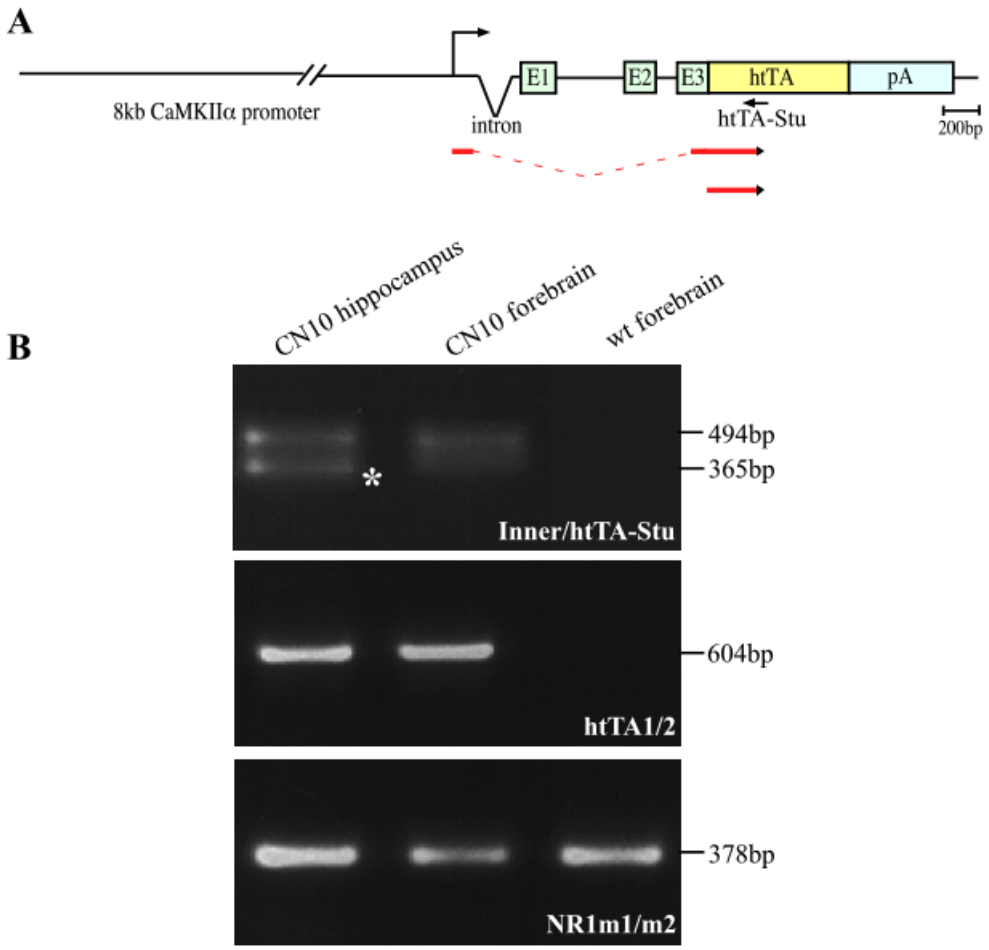

Figure 26. Reverse transcriptase-PCR (RT-PCR) of hippocampus and forebrain in the CN10 line. (A) Schematic representation of the minigene of the CN10. Upper arrow indicates

theoretical transcriptional initiation site driving from CaMKIIα promoter. Down arrow represents primer that sits on the htTA gene and was used for the RT-PCR. Thick red lines show two mRNA forms of the htTA with spliced region (indicated by dashed red line) in CN10 line. (B) RT-PCR fragments on agarose gel. Total RNAs from hippocampus and forebrain of CN10 transgenic lines, respectively, and from forebrain of wild type were amplified by RT-PCR with 5' Capping primer (Inner, see Materials and methods) and htTA-Stu. From hippocampus two fragments (494 bp and 365 bp) were equally amplified compared with forebrain showing weaker band of the small fragment (365 bp, indicated by star). RT-PCR was done with htTA1/2 for the htTA gene and NR1m1/2 for the NR1 gene as a control.

In hippocampal cDNA, the two transcripts of the htTA in the transgenic line CN10 were quantitatively-equally amplified by 5' RACE and analyzed by sequencing (Figure 26). One transcript appears to be derived from the transcription start site of CaMKIIα having 36 bp of CaMKIIα promoter region downstream of transcriptional start and 100 bp of exon 3 out of the 1.0 kb NR2C fragment. The other one harbours only 5 bp upstream of the translational start of the htTA gene. In the forebrain cDNA pool, the long transcript of the htTA was more amplified by 5' RACE, when compared to a relatively equal ratio between the long and short fragment amplified from the hippocampal cDNA.

3. Discussion

Tightly regulated gene expression or knockout in a temporal and spatial manner by reversibly inducible gene regulatory systems should allow a more precise analysis of the impact of a gene function on animal physiology and behaviours. Particularly, we studied the tTA-dependent inducible system, which is one of the currently used regulatory systems for genomic manipulations in transgenic mice. In this study we have described the optimization of tTA for efficient expression in the mouse and establishment of tTA-dependent inducible system by conditional means in transgenic mice.

3.1. The humanized tTA

In previously generated tTA encoding transgenic mice there was often lack of functional tTA expression in the expected tissue. In some of these cases the tTA transcript was found to be incorrectly spliced. To exclude such negative effects on tTA expression by its chimeric prokaryotic and viral gene origin, the coding sequence of tTA was altered, concerning mammalian codon usage, putative splicing signals, CG dinucleotide content and toxicity of the transactivation domain VP16. These sequence changes increased the doxycycline controlled tTA activity three-fold in transiently transfected HeLa cells when compared to the original tTA. Thus the improved tTA (htTA) might serve as an efficient inducible element for the generation of conditional mouse models.

3.2. Transgenic mice expressing htTA

The efficiency of htTA *in vivo* was also evaluated in transgenic mice. For gene transfer of tTA genes BACs were used as a vector, since BACs are expected to provide less integration dependence of transgene expression due to a large size of DNA artificially inserted into the mouse genome. The htTA and the conventional tTA, respectively, were inserted into exon1 harbouring translational start codon of the mouse KA1 gene on one BAC, 166 kb in size. This BAC provided KA1 specific expression of Cre recombinase to transgenic mouse brains before (Shimshek et al. 2001) and now tTA expression was expected to be specific to hippocampal layer CA3 in adult mice (Kask et al.2000). Unfortunately, neither KA1-htTA nor KA1-tTA BAC transgenes activated expression of lacZ indicator genes in tTA-responder lines. However, RT-PCR analysis revealed that from both transgenes htTA and tTA mRNA are expressed respectively and that htTA mRNA was more frequently detected in different transgenic lines when compared with conventional tTA. Thus the expression of htTA and tTA failed to induce detectable expression of tTA-responsive genes in

transgenic mice. In summary, these results indicated that certain amounts of tTA molecules are required for functional activity of the tTA in brain cells and that the KA1 promoter carried by the 166 kb BAC might not be a promoter strong enough for physiological tTA regulated gene expression.

Other promoter elements for brain specific expression were used more successfully. Thus in a subsequent experiment the fusion of the αCaMKII promoter and the silencer fragment of the N-methyl-D-aspatate receptor subunit NR2C gene promoted functional tTA expression in specific cell layers of the hippocampus. Therefore, high levels of tTA are required in neurons, to express tTA-controlled β-galactosidase at detectable levels. Two transgenic lines (sCN1 and CN10), out of 8 analyzed so far, showed a highly restricted expression pattern of the htTA in hippocampus (e.v. Granule cells of the dentate gyrus in mice of line sCN1 at P28, and CA1 pyramidal cells and in granule cells of the dentate gyrus of the CN10 at P50). In other brain regions, which usually show CaMKIIα promoter-mediated expression of a transgene including olfactory bulb, cortex, and striatum of the sCN1 and CN10 (Krestel et al. 2001), tTA levels were observed too low to drive β-galactosidase expression. It is possible that the extraordinary region-specificity of transgenic expression driven by the hybrid NR2C/CaMKIIα promoter in CN10 and sCN1 might result from integration events of a transgene into the genome by accidence, as experienced by others for the CaMKIIα promoter itself (Tsien et al. 1996). On the other hand, it is likely that htTA expression under the CaMKIIα promoter is restricted by the silencer region of the NR2C gene in sCN1 and CN10 derived mice. First, due to statistic frequency of appearance of highly restricted expression pattern, 2 out of 8. Second, judging from several experiments in cell culture and transgenic mice, the NRSE always functions as a silencer of transcription in non-neuronal cells (Millecamps et al. 1999, Bessis et al. 1997, Roopra et al. 2000, Suchanek et al. 1997, Mieda et al. 1996). Three, it has been shown that the expression of a NRSE-controlled expression via adenoviral vectors was effectively controlled in neuronal cells, with a perfect suppression in non-neuronal cells (Millecamps et al. 1999). In addition, in the study of Suchanek et al. (1997), the 1.0 kb silencer fragment including untranslational exons1-3 of the NR2C gene was identified as a important regulatory region for NR2C-specific expression by negatively regulating the transcription in NR2C negative cells.

The chimeric NR2C/CaMKII promoter shows strongest htTA expression in granule cells of the dentate gyrus in both lines sCN1 and CN10. Especially in CN10, β-galactosidase signals showed high intensity in granule cells of the dentate gyrus compared to CA1 pyramidal cells. This suggests that htTA expression might be promoted by the NR2C silencer as well as the CaMKIIα promoter because the expression of NR2C and α-CaMKII overlaps in this cell type only.

However, in one line (CN4) no effect of the NR2C silencer was found. In these animals the htTA expression pattern was very similar to CaMKIIα promoter driven transgenes. A mRNA analysis and detailed analysis of transgene might help explain the exception. In any case CN4 mice underline the integration-dependent variety of expression pattern which are frequently obtained in different transgenic lines.

3.3. Suppression and reinduction in the CN10 by doxycycline

As observed for CaMKII-tTA (Krestel et al. 2001) the tTA driven induction of indicator genes after removal of doxycycline did not reach its expected value even three weeks after doxycycline removal for line CN10.

Thus failure to reach full β-galactosidase might result from storage of doxycycline in tissues during three weeks of early postnatal doxycycline treatment, and from slow clearance of doxycycline in mature brain. However, mice using rtTA have a similar problem (Hasan et al. 2001). The incomplete induction, which can not be resolved even by six months of doxycycline treatment. Therefore the incomplete reinduction seems not to be a problem of tTA activation but might be generated by the indicator genes.

Similar to CaMKII-tTA expression pattern the CN10 mice with incomplete tTA induction in the pyramidal neurons of CA1 show heterogenous levels of β-galactosidase expression, with cells showing weak and no β-galactosidase expression (Krestel et al.2001). Thus variability and mosaic β-galactosidase expression in CA1 pyramidal cells in reinduced $Tg^{CN10/MNL}$ can not entirely be explained by slow kinetic of doxycycline clearance only. If cells of same type, e.g. pyramidal cells of CA1, were theoretically under the same condition at least in terms of intracellular doxycycline and tTA level, only homogenously decreased expression level of β-galactosidase by removal of doxycycline should be observed within same type cells.

In doxycycline-untreated $Tg^{CN10/MNL}$, it could be possible that tTA-responsive β-galactosidase is expressed at different level in CA1 pyramidal cells and granule cells of dentate gyrus, but it could not be visualized by X-gal staining and immunocytochemistry because the signals were too strong to distinguish variable intensity. However, the differences become apparent by doxycycline treatment. Then activity of htTA and β-galactosidase expression are down-regulated by administration of doxycycline and variable intensity of β-galactosidase signals appears in distinct CA1 pyramidal cells. In granule cells of dentate gyrus the saturated different signal strengths can not be distinguished.

The molecular basis for the heterogenous expression remains obscure but it might also be possible that the brain has such a heterogeneous intrinsic condition of cell context as asynchronous responsibility to doxycycline or gene expression level and expression

timing even within same type cells e.g. pyramidal cells of CA1. It has been observed that certain endogenous genes are expressed with a stochastic pattern (Van Roon et al. 1989 and Ko 1992). In the study of Van Roon et al. (1989), the intracellular heterogeneity within individual embryonic hepatocytes was investigated by studying the ratios of accumulation of endogenous carbamoylphosphate synthetase, phosphoenolpyruvate carboxykinase, and arginase with time. It was suggested that the stochastic gene expression in developing hepatocytes is caused by competition of different genes for an initially limiting supply of common regulatory factors, leading to random differences in the rate of accumulation of respective gene products. The mosaicism frequently observed in transgenic mice may result from the heterogeneity of the individual cell contexts, in spite of the physiological homogeneity of the same type cells.

3.4. Future applications

Another invaluable approach for conditional gene regulation is the improvement of temporal control of the Cre-loxP system by combining the Cre-loxP system with the tTA-dependent inducible system. The Cre-mediated site-specific recombination via loxP sites is currently a reliable, powerful technique of genomic manipulation. The Cre-mediated intra-allelic recombination has been successfully established in transgenic mice but is irreversible. However, if the Cre recombinase is active at early developmental stage, it irreversibly excises the gene segment flanked by loxP sites, at undesired time points, resulting in limitations for the analysis. Therefore, the activity of Cre recombinase should be regulated in selected regions and at chosen time. As described above, the tTA-mediated regulatory system is reversible but by withdrawal of doxycycline the transgene was heterogeneously expressed. Compensation of drawbacks of two systems by combining both systems will ultimately provide a precise control of Cre activation in the brain.

There is a transgenic line called LC1 containing a tTA-responsive Cre recombinase and a luciferase gene in a bidirectional module (Hasan et al 2001). In LC1, the Cre recombinase expression can be spatially regulated by the particular promoter used for the tTA or rtTA and temporally by treatment with doxycycline. Then, Cre recombinase will be expressed in particular cell populations and will mediate site-specific recombination in the loxP-flanked gene at chosen developmental time. In particular, it can be expected by combining CN10 and LC1 that spatial control of the Cre recombinase expression is achieved by CN10, namely in CA1 pyramidal cells and dentate gyrus granule cells only. Its temporal control can be achieved at any time by doxycycline removal. Thereby, the hippocampus-restricted knockout of genes of

interest could be achieved and lead to a more precise analysis of the impact of a gene mutation on animal behaviour.

3.5. Conclusion

The improved tTA (htTA) provides efficient expression and reversible induction *in vitro* and *in vivo*. A chimeric promoter, consisting of the CaMKIIα promoter and the NR2C silencer, has been generated to direct htTA expression to selected cell populations in the hippocampus. It was demonstrated that the chimeric NR2C/CaMKIIα promoter could provide highly restricted gene expression in transgenic mice. The new htTA lines with exquisite hippocampal expression and postnatal initiation should help to address questions concerning the specific role of the hippocampus in behaviour.

4. Materials and Methods

4.1. Synthesis and cloning of the htTA

The humanized tTA (htTA) was designed at the DNA level without altering amino acids sequences of the prokaryotic one. The htTA was synthesized according to the method of Stemmer et al. (1995). Twenty oligonucleotides (S1-S10 and C1-C10), which overlap on the designed htTA DNA sequence and are approximately 60 nucleotides in length encoding the htTA were purified with POLY-PAK™ cartridge (GLEN research) and then assembled by PCR with 5 u of pfu polymerase (Stratagene); 55 cycles at 94 ^{o}C for 30 sec, 52 ^{o}C for 30 sec, and 72 ^{o}C for 30 sec. The htTA was amplified from the assembled PCR pool with two outside primers (T-front and T-back) containing restriction enzyme sites, EcoRI and BamHI respectively. The PCR program of amplification consisted of 23 cycles at 94 ^{o}C for 30 sec, 50 ^{o}C for 30 sec, and 72 ^{o}C for 60 sec. The synthesized htTA fragment (784 bp) was digested with EcoRI and BamHI and cloned pBluscriptIISK+ (Stratagene), called pBS.htTA+nls. To express the htTA gene and the prokaryotic one (pUHD 15-1, Gossen and Bujard 1992) in cells, they were subcloned into expression vector pRK5 (Schall et al. 1990) via EcoRI/BamHI digestion.

4.2.Cell culture, transient transfections and luciferase assay

HeLa X1/6 cells containing chromosomally integrated luciferase reporter gene were maintained at 37°C and 5 % CO_2 in Earl's modified Eagles medium (E-MEM, Gibco) supplemented with 10 % Fetal Calf serum (Baron et al. 1997). After 30 hours of co-transfection of 5 μg of pRK5.htTA-nls, pRK5.htTA+nls, pRK5.tTA, respectively and 5 μg of pCMVpnlacF (Mercer et al. 1991) by calcium phosphate transfection according to standard protocols, transfected HeLa X1/6 cells were homogenized in lysis buffer (100 mM potassium phosphate pH7.8, 0.2 % Triton X-100, 0.5 mM DTT) according to manufacture's protocol (TROPIX). Homogenate was measured in a luminometer for luciferase activity by use of the luciferase gene assay kit (Dual-Light, TROPIX). Luciferase activity was normalized to β-galactosidase activity assayed either simultaneously by luminescent assay or by standard liquid o-Nitrophenyl-β-D-Galactopyranoside (ONPG, Sigma) assay (Miller 1972-Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, NY).

4.3. Screening of BACs library for isolation of mouse KA1 BACs

The 682 bp of PCR fragment, covering the exon1 harbouring ATG and partial introns surrounding the exon1of the mouse KA1 gene, was amplified with KA5up and

KAY2. This amplified fragment was used for screening of the BAC library of the mouse C57BL/6 strain (Genome Systems, St. Louis, MO, USA) and isolation of two mouse KA1 BACs (approximately 166 kb and 60 kb).

4.4. Pulsed field gel eletrophoresis (PFEG)

BAC DNA were isolated according to standard plasmid preparation and digested with several endonucleases (e.g. NotI). Digested BAC DNA separated on 1 % agarose gel in TAE buffer (40 mM Tris/acetate and 1mM EDTA) by PFGE using the following conditions: 1 sec initial switch time, 6 sec final switch time, 6 V/cm field strength, 120 ^{o} angle, for 16 hours at 14 ^{o}C (BioRad CHEF III, BioRad, Hercules, CA, USA). After PFEG the gel was stained with ethidium bromide.

4.5. Construction of targeting cassettes of the htTA and tTA

Targeting cassette contains following elements (5´ to 3´); recombinogenic arm (RA) A, the htTA or tTA, a polyadenylation sequence (pA) of human growth hormone gene, and FRT-flanked selection marker (KM gene) and recombinogenic arm B. The targeting cassette was constructed by serial cloning steps. Two recombinogenic arm A and B were amplified with KA1 5´up/newKA3 (for A, 630 bp) and KA1-3´Sal/3´newKA1 (for B, 560 bp) respectively from 166 kb KA1 BAC. To substitute translational start codon of the htTA or tTA for that of the KA1, XbaI site next to ATG codon was introduced by PCR mutagenesis (pBS.htTA+nls, pUHD 15-1). Selection marker, kanamycine resistant gene from pPKM6 (Reiss et al. 1984) was flanked by FRT sites via insertion of linker oligos containing FRT site and restriction sites (Xho-FRT-HincII/comXho-FRT-HincII and EcoRI-FRT-SacI/comEcoRI-FRT-SacI) and subcloned into pBluscriptIISK+ (Stratagene), called pBS.KM6-FRT. The targeting cassette was serially subcloned into pLitmus 28 (New England Biolab) via following digestions (5´ to 3´); RA A (KpnI/XbaI), htTA or tTA (XbaI/BamHI), pA (BamHI/XhoI) from pBS.hghpA, FRT flanked KM gene (XhoI/SacI), and RA B (SacI/BglII).

4.6. Modification of BACs through homologous recombination

(1) Subcloning into shuttle vector and first recombination

The targeting cassette (approximately 4 kb) was released from pLitmus 28 vector backbone and then cloned into NotI induced temperature-sensitive shuttle vector (pSV1.recA) via NotI digestion (Yang et al. 1997). The shuttle vectors carrying targeting cassette (pSV1.recA-htTA and -tTA) were transformed into the DH10B

containing the 166kb KA1 BAC. The transformants were plated on 10 μg/ml tetracycline (by pSV1.recA), 12.5 μg/ml chloramphenicol (by BAC plasmid) and 25 μg/ml kanamycine (by targeting cassette) plates at 30°C overnight. After first homologous recombination between the shuttle vector and the BAC, cointergrates were digested with BamHI and identified by southern blot with RA A and B as probe.

(2) Second recombination of cointegrates

Cells containing correct cointegrate were incubated on plates containing 12.5 μg/ml chloramphenicol and 25 μg/ml kanamycine at 43°C overnight. Subsequently, cells were replated on 12.5 μg/ml chloramphenicol, 25 μg/ml kanamycine, and Fusaric acid (16 μg/ml) plates at 30°C for two days. Resolved BACs were detected by colony PCR with outside primers newKA1-30 and 3´newKA1; denature at 96 ^{o}C for 10 min, followed by 35 cycles of 94 ^{o}C denature for 20 sec, 60 ^{o}C anneal for 30 sec, and 72 ^{o}C extension for 3 min. BamHI-digested resolved BAC were verified by southern blot with RA A and B as probe.

(3) Flp recombination

Chlroamphenicol resistant maker of plasmid encoding Flp recombinase (pMAK-705Flp; Buchholz et al.1996) was substituted for ampiciline resistant gene, pMAK-705Flp.amp) and transformed into cells containing the resolved BAC. Transformants were plated on both 100 μg/ml ampiciline (by pMAK-705Flp.amp), 12.5 μg/ml chloramphenicol plates at 30°C overnight, whereupon colonies were replated on only 12.5 μg/ml chloramphenicol plates at 40°C for loss of temperature sensitive Flp plasmid. Flp recombinants were identified by colony PCR with outside primers newKA1-30 and 3´newKA1and by southern blot analysis with RA A and B as probe.

4.7. Purification of the linear BAC DNA for pronuclear injection and identification by tail biopsy PCR

The modified BAC DNA (30 μg-50 μg) were digested with NotI in reaction buffer including 2.5 mM spermidine (Sigma). Digested BAC DNA mixed with gel loading buffer were run through the sepharose CL-4B (Pharmacia) column, which was equilibrated with 30ml of the microinjection buffer (10 mM Tris/HCl pH7.5, 0.1 mM EDTA and 100 mM NaCl). Fractions of 500 μl were collected and small volume of every fractions was identified on a 1 % agarose pulsed field gel. The appropriate fractions with intact BAC DNA and no vector bands were adjusted appropriate DNA concentration (1μg/ml) and were injected into pronucleus of C57BL/6 mice zygote (ZMBH, INF 282, D-69120 Heidelberg, Germany). Transgenic mice were identified

by PCR analysis of mouse tail DNA using BAC R1/2 amplifying a 300 bp fragment of BAC terminal and htTA1/2 amplifying a 604 bp fragment of htTA.

4.8. cDNA synthesis and RT-PCR

Total RNA was isolated from mouse brains using TRI REAGENT (Molecular Research Center, Inc., USA) according to manufacturer's protocols. Approximately 5 μg of total RNA was reverse transcribed for 1 h at 37 ^{o}C with 200 u reverse transcriptase (MMLV; BRL) in a 20 μl reaction containing 10 mM dithiothreitol, 3 mM $MgCl_2$, 20 u RNAsin (Fermentas), 0.5 mM dNTP and random hexamer (dN_6; Boehringer Mannheim). Mock cDNA was performed except reverse transcriptase. 1 μl of reverse transcription reaction was used as template for subsequent PCR reaction with primer sets of htTA1/2 and NRm1/m2 under a condition; denature at 96 ^{o}C for 5 min, followed by 30 cycles of 94 ^{o}C denature for 30 sec, 60 ^{o}C anneal for 30 sec, and 72 ^{o}C extension for 30 sec. PCR products were visualized on 1.5 % agarose gel stained with ethidium bromide.

4.9. Construction of minigenes, sCNhtTA and CNhtTA

The CNhtTA minigenes contains 8.5 kb fragment of the CaMKIIα promoter, 1.0 kb of the NR2C exons 1-3 (silencer), the htTA, and a polyadenylation signal of human growth hormone (pA). 1.0 kb of silencer (SmaI/SalI from p1.0nlacF; Suchanek et al. 1997) and the htTA expression unit with pA (SalI/EcoRI) were cloned into pBluscriptIISK+ (SmaI/EcoRI) by three fragments ligation, pBS.1.0NhtTA. The 1.0NhtTA fragment was released via SmaI/EcoRV digestion, inserted into pNN265 (Mayford et al. 1996) via EcoRV digestion and subsequently cloned into pMM403 (Mayford et al. 1996) via NotI digestion. 11 kb of CNhtTA minigene was released by Srf restriction.

The sCNhtTA minigene consists of 0.4 kb fragment of the CaMKIIα promoter, 1.0 kb of the NR2C exons 1-3 (silencer), the htTA, and a polyadenylation signal of human growth hormone (pA). 0.4 kb CaMKIIα promoter fragment (KpnI/EcoRI) and 1.0 kb of silencer (EcoRI/SalI from p1.0nlacF) were inserted into pBS.htTApA opened by KpnI/SalI. 3.4 kb of sCNhtTA minigene was released by DraIII/BglII digestion.

4.10. Purification and identification of transgenes, sCNhtTA and CNhtTA

10 μg of linear DNA of sCNhtTA (3.4 kb) and CNhtTA minigene (11 kb) were separated by sucrose density gradient centrifugation in a Beckman L8-70M ultracentrifuge using an SW40T rotor (10 %-40 % sucrose, 35000 rpm, 16 h at 15 ^{o}C). Appropriate fractions containing the minigenes without vector backbone were purified by Amicon Micron 50 Filters (Millipore). Transgenic mice were identified by

PCR analysis of mouse tail DNA using htTA1 and htTA2 amplifying a 604 bp fragment. Transgenic mice generated by pronucleus injection of C57Bl/6 derived oocytes with 1 ng/μl of the constructs DNA.

4.11. X-gal staining for vibratome sections

Brains were removed and fixed for 2 h in 4 % Parformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4/2H_2O$, 1.4 mM KH_2PO_4). Afterwards the brains were rinsed wit PBS, embedded in 2% agarose (Seakem LE) in PBS and cut sagittally or coronally in 100 μm sections on a vibratome (Leica VT 1000S, Leica). The sections were incubated for 5-60 min at room temperature in X-Gal staining solution (5 mM $K_4Fe(CN)_6$, 5 mM $F_3Fe(CN)_6$, 2 mM $MgCL_2$, 2 mg/ml X-Gal in dimethylformamid/PBS). Sections were washed twice in PBS and once 10 mM Tris/HCl, pH7.6. Sections were immediately counterstained with eosin (Sigma) for 1 min and rapidly and successively dehydrated in ethanol 70, 90, 99.5 % (v/v). The dry sections were dehydrated in Xylene and embedded in EuKitt (Kindler GmbH, Germany).

4.12. Immunocytochemistry (ICC)

Mice were anesthetized with halothane (Hoechst, Germany) and intracardially perfused with phosphate-buffered saline, pH 7.4 (PBS), at room temperature followed by ice-cold 4 % paraformaldehyde (PFA) in PBS. Brains were removed and post-fixed for 1 h in PBS containing 4 % PFA at 4°C. Afterwards the brains were rinsed with PBS and embedded in 2 % agarose in PBS and cut sagittally or coronally in 100 μm sections on a vibratome (Leica VT 1000S; Leica, Germany). For blocking of endogenous peroxidase, sections were incubated for 10 min in 0.5 % H_2H_2/PBS and washed twice for 10 min with PBS. Sections were permeabilized in Day 1 buffer (0.3 % Triton X-100, 1 % bovine serum albumine (BSA), 3 % normal goat serum in PBS) and incubated overnight in buffer 1 containing anti-β-galactosidase rabbit IgG (1:8000; ICN, Germany). The following day, sections were washed twice for 10 min in Day 2 buffer (0.1% Triton X-100 and 0.3 % BSA in PBS) and incubated for 1 h in Day 2 buffer supplemented with peroxidase-conjugated anti-rabbit IgG (1:600; Vector Laboratories, USA), washed for 10 min in Day 2 buffer , twice for 10 min in PBS. The staining reaction was developed using 20mg Diaminobenzidine (DAB; Sigma) dissolved in 50 ml of 20 mM Tris/HCl pH7.6 and stopped in PBS. After a brief rinse sections were mounted Eukitt (Kindler GmbH, Germany).

4.13. Doxycycline treatment to mice

Doxycycline hydrochloride (Sigma) at a concentration of 50 μg/ml, supplemented with 1 % sucrose, was dissolved in drinking water and provide to the parental mice in light-protected bottles. Animals were kept under doxycycline from P1 and until P21 (or P42). At P21 doxycycline was removed from the drinking water.

4.14. Cryostat section

Mouse brains were removed and frozen. Coronal sections of 15 μm in thickness were cut on a cryostat. The section were immediately fixed for 5 min in PBS containing 2 % PFA and rinsed with PBS. For X-gal staining sections were incubated for 5 min in X-gal solution and afterwards washed twice in PBS and once in water. Sections were immediately counterstained with red neutral (Sigma) for 3 min and rapidly and successively dehydrated in ethanol 70, 90, 99.5 % (v/v). The dry sections were embedded in Eukitt (Kindler GmbH, Germany). For ICC sections were performed according to previously described protocol.

4.15. RT-PCR and RACE (rapid amplification of cDNA ends)

Total RNA was isolated from hippocampus and forebrain of the CN10 mice and RT-PCR was done with primer sets of htTA1/2 and NR2m1/m2. Amplification of the 5´-end of a transgene, the htTA, was performed with FirstChoice RNM-RACE kit (Ambion) according to manufacturer's protocols, using 5 μg of total RNA from hippocampus and forebrain. The outer adapter primer and outer htTA-specific primer (htTA2) were used for the first nested PCR under a condition; denaturate at 94 ^{o}C for 5 min, followed by 35 cycles of 94 ^{o}C denature for 30 sec, 60 ^{o}C anneal for 30 sec, and 72 ^{o}C extension for 30 sec. A second nested PCR followed with inner adapter primer and inner htTA-specific primer (htTA-Stu). The PCR products were subcloned into pCR4 vector (Invitrogen) and sequenced.

For the applied standard molecular biological techniques refer to;
Current Protocols in Molecular Biology : Ausubel, Brent, Kingston, Moore, Seidman, Smith, Struhl, Wiley Interscience, 1989
Molecular Cloning, A Laboratory Manual : Sambrook, Fritsch, Maniatis, 2nd Edt., Cold Spring Harbor Laboratory Press, 1989

4.16. Sequences of used oligonucleotides (5´-3´)

(1) Synthesis of htTA

S1: ATGTCCAGACTGGACAAGAGCAAAGTCATCAACTCTGCCTTGGAGCTCCTGAATGAAGTT
S2: CCACCAGGAAGCTGGCCCAGAAGCTGGGTGTGGAGCAGCCTACCCTGTACTG
S3: CAAGAGGGCTCTGCTTGATGCCCTGGCCATTGAGATGTTGGACAGGCACCACACCCAC
S4:AGGGGAGTCCTGGCAGGACTTCCTGAGGAACAATGCCAAGAGCTTCAGATGTGCCTTGCTCTCCCACC
GG
S5: CACCAGGCCTACAGAGAAGCAGTATGAGACCCTGGAGAACCAGCTGGCATTCCTGTGCC
S6: AAATGCCTTGTATGCCCTCTCTGCTGTGGGCCACTTCACCTTGGGCTGTGTGCTGGAGG
S7: AAGGAGGAGAGGGAGACCCCCACCACTGACTCCATGCCACCACTGCTGCGGCAAGCT
S8: GGGGCTGAGCCTGCATTCCTTTTTGGCCTGGAACTGATCATCTGTGGCCTGGAAAAGCAG
S9: TCACGTGCCCAAAAAGAGAAAGCACGTGCCTGCTGATGCCTTGGATGATTTTGACCTGGA
S10:CTGGATGACTTTGATTTGGACATGCTCCCTGCTGATGCACTTGATGATTTTG

C1: TCACAGCATATCCAGGTCAAAATCATCAAGTGCATCAG
C2: GTCCAAATCAAAGTCATCCAGGGCATCAGCAGGCAGCATGTCCAGGTCAAAATCATCCAA
C3: TTCTCTTTTTGGGCACGTGAGAGCCAGACTCACATTTCAGCTGCTTTTCCAGGCCACAG
C4: GGAATGCAGGCTCAGCCCCTTGGTGGTCAAACAACTCAATAGCTTGCCGCAGCAGTGGTG
C5: GGTCTCCCTCTCCTCCTTGGCAACTTGGTGCTCCTGGTCCTCCAGCACACAGCCCAAG
C6: GAGAGGGCATACAAGGCATTTTCCAGGGAGAAGCCTTGTTGGCACAGGAATGCCAGCTGG
C7: GCTTCTCTGTAGGCCTGGTGCCCAAGTGAACTTTGGCACCATCCCGGTGGGAGAGCAAGGC
C8: TGTTCCTCAGGAAGTCCTGCCAGGACTCCCCTTCCAGAGGGCAGAAGTGGGTGTGGTGCCTGTC
C9: CATCAAGCAGAGCCCTCTTGTTCTTCACATGCCAGTACAGGGTAGGCTGC
C10:GGGCCAGCTTCCTGGTGGTCAAGCCCTCAATGCCAACTTCATTCAGGAGCTCC

T-front GGCGAATTCACCATGTCCAGACTGGACAAG
T-back CGCGGATCCATCACAGCATATCCAGGTC

(2) KA1 related oligonicleotides

KA5up	TCTCGATTCCTAACTAGAG
KAY2	AACCTGTCATGAATCCTACCG
newKA1-30	AGTGCCTCTGGCAGCTGAAGCAGGTGCACA
3'newKA1	TGTCTTCCATTTCAGAGCTATGCCATGGCT
newKA3	AGACTCTAGACATCTTCTATAACTCCTCATGGAGCCC
KA1-3´SalI	TAATGTCGACCCGTGTCTCTGCTCCTCTGGTGCTG
BAC R1	GTGTCACCTAAATAGCTTGGCG
BAC R2	GGGGTTCGCGTTGGCCGATTC

(3) FRT site

Xho-FRT-HincII	TCGAGGAAGTTCCTATACTTCTAGAAGAATAGGAACTTCGTC
comXho-FRT-HincII	GACGAAGTTCCTATTCTTCTAGAAGTATAGGAACTTCC
EcoRI-FRT-SacI	AATTCGAAGTTCCTATACTTCTAGAAGAATAGGAACTTCGAGCT
comEcoRI-FRT-SacI	CGAAGTTCCTATTCTTCTAGAAGTATAGGAACTTCG

(4) RT-PCR and RAC

htTA1	AGAGCAAAGTCATCAACTCTGCC
htTA2	GTGAGAGCCAGACTCACATTTCA
NR1m1	AACTGCAGCGTGCGCAGTACATAGAG
NR1m2	CGGAATTCCAGCCCACACCATGCCTAG
htTA-Stu	TCTGTAGGCCTGGTGCCCAAGTGAACTTTG
outer adapter primer	GCTGATGGCGATGAATGAACACTG
inner adapter primer	CGCGGATCCGACACTCGTTTGCTGGCTTTGATG

(5)Sequencing primers

T3-25	CGCGCAATTAACCCTCACTAAAGGG
T7-24	CGTAATACGACTCACTATAGGGCG
lac	GTTTTCCCAGTCACGAC
lac reverse	CAGGAAACAGCTATGAC
CIS3	GTAACCATTATAAGCTGC
SP6-N	TACGATTTAGGTGACAC

4.17. Abbreviations

AMPA	α-amino-3-hydroxy-5methyl-4-isoxazole-propionic acid
APRT	adenine phosphoribosyltransferase
BAC	bacterial artificial chromosome
BDNF	brain-derived neurotrophic factor
bp	base pair
CA	areas of Ammon's horn
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cDNA	complementary deoxyribonucleic acid
CRF	corticotropin-releasing factor
DG	dentate gyrus
DNA	deoxyribonucleic acid
FRT	Flp recombinase target site
GABA	γ-aminibutyric acid
GFP	green fluorescent protein
h	hours
htTA	humanized tetracycline-controlled transactivator
KA1	high-affinity kainate receptor subunit 1
kb	kilobase pair
min	minutes
mRNA	messenger ribonucleic acid
nls	nuclear localization signal
NMDA	N-methyl-D-aspatate; Ach, acetylcholine
NRSE	neuron-restrictive silencing element
P	postnatal day
PCR	polymerase chain reaction
PFEG	pulsed-field gel electrophoresis
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
sec	seconds
Tg	transgenic mouse
tTA	tetracycline-controlled transactivator
VGF	vascular endothelial growth factor
X-gal	5-Brom-4-Chlor-3-Indolyl-b-D-Galactopyranoside
YAC	yeast artificial chromosome

5. References

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